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## Genetic and Biological Markers of Severity in Sickle Cell Disease

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# Genetic and Biological Markers of Severity in Sickle Cell Disease

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Submitted for degree of Doctor of Philosophy

## Abstract

Sickle cell disease (SCD) is remarkable for the variability of its phenotype despite its genetic simplicity, with family and population studies indicating a strong genetic influence. The best characterised genetic modifiers are genes controlling HbF levels and co-inheritance of alpha-thalassaemia. Other genetic and biological factors may also influence disease severity and the development of complications.

King's Health Partners have the largest adult cohort of SCD patients in the United Kingdom, approximately 2400 patients. Characterising this group (using laboratory variables, evidence of clinical complications and admission data) has formed the basis for my studies. Telomere length and Duffy antigen receptor for chemokines (*DARC*) status and two complications, sickle nephropathy and sickle hepatopathy were investigated.

Relative telomere length (measured using qPCR) was significantly longer in patients with SCD than controls and positively correlates with white blood count. Shorter telomeres were found in patients on hydroxycarbamide treatment and those with Hb SC. We hypothesise that longer telomeres result from up-regulation of telomerase due to inflammation.

Polymorphisms in *DARC* have a high prevalence in people of African ancestry and explain benign ethnic neutropenia. We found positive associations with Duffy positive phenotype and a reduced time to readmission and the development of leg ulcers.

We investigated if sickle-related renal impairment was associated with the *APOL1*, *DARC* and *HMOX1* genes. Duffy positive phenotype was associated with the development of macroalbuminuria and the presence of 1 or more *APOL1* risk alleles was associated with the development of renal impairment (as measured by MDRD eGFR).

We assessed the prevalence of liver disease in our population using Enhanced Liver Fibrosis score (a combination of serum markers associated with liver fibrosis) and transient elastography (Fibroscan®). Using this approach we found that transfusional iron overload and haemolysis appear to play a key role in the pathogenesis of sickle hepatopathy.

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## Table of abbreviations

Abbreviation	Meaning
AA	Aplastic anaemia
ACE	Angiotensin converting enzyme
ACR	Albumin to creatinine ration, a measure of renal function
ACS	Acute chest syndrome
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AML	Acute myeloid leukaemia
AST	Aspartate transaminase
AVN	Avascular necrosis
BMT	Bone marrow transplantation
cGMP	Cyclic guanosine monophosphate
CKD	Chronic kidney disease
CT	Computerised tomography
CVA	Cerebrovascular accident
<i>DARC</i>	Duffy antigen receptor for chemokines
DC	Dyskeratosis congenita
eGFR	Estimated glomerular filtration rate
ELF score	Enhanced liver fibrosis score
EPO	Erythropoetin
EPR	Electronic patient record
ES	Erythroid silent
ESRD	End stage renal disease
FSGS	Focal segmental glomerular sclerosis
Fy	Duffy
GFR	Glomerular filtration rate, a measure of renal function
GGT	Gamma-glutamyl transpeptidase
Glu	Glutamic acid
GTP	Guanosine triphosphate
GvHD	Graft versus host disease
GWAS	Genome wide association study
HAI	Histology activity index
Hb	Haemoglobin
Hb SC	Hb SC disease (compound heterozygous inheritance of HbS and HbC)
Hb SHPFH	Compound heterozygous inheritance of HbS and HPFH
Hb SS	Homozygous inheritance of HbS
HbC	Haemoglobin C
HbE	Haemoglobin E
HbF	Haemoglobin F (foetal haemoglobin)
HbS	Haemoglobin S (sickle haemoglobin)
HbS $\beta$ thalassaemia	Compound heterozygous inheritance of HbS and Hb $\beta$ + or Hb $\beta$ 0
HC	Hydroxycarbamide/hydroxyurea
HDACi	Histone diacetylase inhibitors
HIV	Human immunodeficiency virus

Abbreviation	Meaning
HLA	Human leukocyte antigen
HPFH	Hereditary persistence of foetal haemoglobin
IPH	Idiopathic pulmonary hypertension
KCH	King's College Hospital
KDOQI	Kidney Disease Outcomes Quality Initiative
LDH	Lactate dehydrogenase
Lys	Lysine
MCH	Mean cell haemoglobin
MCV	Mean cell volume
MDRD eGFR	Modification of diet in renal disease estimated glomerular filtration rate
MDS	Myelodysplastic syndrome
MMqPCR	Multiplex quantitative polymerase chain reaction
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
N/A	Not applicable
NCEPOD	National confidential enquiry into patient outcomes and death
NCT	National clinical trial
NIH	National institute of health
NO	Nitric oxide
PCR	Protein to creatinine ratio
PHT	Pulmonary hypertension
POT1	Protection of telomeres 1
pRB	Retinoblastoma protein
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
RBC	Red blood cell
SCA	Sickle cell anaemia (HbSS and Sp <sup>0</sup> )
SCD	Sickle cell disease (all genotypes involving homozygote or heterozygote inheritance of haemoglobin S causing a clinical phenotype)
SCLD	Chronic sickle lung disease
SCN	Sickle cell nephropathy
SD	Standard deviation
SNP	Single nucleotide polymorphism
sTR	Soluble transferrin receptor
TAPS	Transfusion perioperatively in sickle cell disease
TCD	Transcranial dopplers
TERC	Telomerase RNA component
TERT	Telomere reverse transcriptase
TIA	Transient ischaemia attack
TIBC	Total iron binding capacity
TRJet	Tricuspid regurgitant jet
UNK	Unknown
VCAM-1	Vascular cell adhesion molecule-1
WBC	White blood cell count

## **Contribution to Thesis**

### **Chapter 3: King's Sickle Cohort**

Design of data collection sheets

Contribution to design of database fields (with Prof Thein)

Data collection, verification and validation (with Prof Thein)

Data entry onto database (with Dr Andrew Hearn and Prof Thein)

Statistical analysis

### **Chapter 4: Leucocyte Telomere Length in Sickle Cell Disease**

Recruitment of patients to DNA gene bank (with Prof Thein)

Extraction of DNA samples (with Dr Vasavda and Helen Rooks)

Collection of clinical and laboratory data

Method development

Measurement of telomere length (with Dr Jie Jang)

Statistical analysis

### **Chapter 5: The Effect of Duffy Antigen Receptor for Chemokines (DARC) on Severity in Sickle Cell Disease**

Recruitment of patients to DNA gene bank (with Prof Thein)

Extraction of DNA samples (with Dr Vasavda and Helen Rooks)

Collection of clinical and laboratory data

Genotyping of gene bank samples for the *DARC* promoter polymorphism (C/T at -33)

Statistical analysis (with Dr Tony Fulford)

## **Chapter 6: Renal Impairment in Sickle Cell Disease**

Recruitment of patients to DNA gene bank (with Prof Thein)

Extraction of DNA samples (with Dr Vasavda and Helen Rooks)

Genotyping gene bank samples for the *DARC* promoter polymorphism (C/T at -33), presence or absence of the *APOL1* G1 risk polymorphism (G) and the *APOL1* G2 risk allele (del TTATAA) and microsatellite genotyping of *HMOX1* promoter polymorphism

Confirmation of *APOL1* G2 risk allele using Sanger sequencing

Collection of clinical and laboratory data

Statistical analysis (with Dr Tony Fulford)

## **Chapter 7: The Effect of Sickle Cell Disease on the Liver**

Recruitment of patients to DNA gene bank (with Prof Thein)

Recruitment of patients to liver study

Extraction of DNA samples (with Dr Vasavda and Helen Rooks)

Performing transient elastography (204 scans) with Dr Emer Fitzpatrick (10 scans)

Collection of clinical and laboratory data

Microsatellite genotyping of *HMOX1* promoter polymorphism

Statistical analysis



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# Chapter 1 Introduction

## 1.1 Haemoglobin

### 1.1.1 Structure

Haemoglobin (Hb) is the protein that carries oxygen in red blood cells (erythrocytes) and delivers it to all body tissues where it is exchanged with carbon dioxide which is later expelled via the lungs.

In 1959 Max Perutz determined the molecular structure of haemoglobin using x-ray crystallography. Haemoglobin consists of two  $\alpha$ -like and two  $\beta$ -like globin chains with each chain containing an oxygen carrying heme group (Perutz, *et al* 1960).

Different haemoglobin protein forms are synthesised at different stages of foetal development. It is believed that this occurs due to the changing oxygen demands which occur. A total of six types of haemoglobin exist, and the first three, Gower I ( $\zeta_2\epsilon_2$ ), Gower II ( $\alpha_2\epsilon_2$ ), and Portland ( $\zeta_2\gamma_2$ ) are only produced in the embryo (Huehns, *et al* 1964). The first “globin-switch” occurs from embryonic to foetal haemoglobin ( $\alpha_2\gamma_2$ , HbF) at about 6-8 weeks of gestation. Around birth the second switch is from foetal to adult haemoglobin (Hb A<sub>2</sub>;  $\alpha_2\delta_2$  and Hb A;  $\alpha_2\beta_2$ ). Hb A<sub>2</sub> is a minor form of haemoglobin which makes up 2-3% of total haemoglobin (Kunkel and Wallenius 1955). Hb A represents more than 95% of total haemoglobin (in “normals”) and remains the dominant form of haemoglobin throughout life (Stamatoyannopoulos 1972). The  $\beta$ -globin locus is situated on chromosome 11 and consists of genes coding for  $\epsilon$ -,  $\gamma$ -,  $\delta$ - and  $\beta$ -globin chains. The  $\alpha$ -globin cluster is located at the telomere end of chromosome 16 and consists of a single  $\zeta$ -globin gene and two co-expressed  $\alpha$ -globin genes which encode an identical protein.

### 1.1.2 Haemoglobin disorders

More than 700 haemoglobin variants have been described to date with new discoveries on a yearly basis. These variants can affect a) the quantity or b) the quality of haemoglobin. The  $\alpha$ -globin and  $\beta$ -globin chains can be affected.

Quantitative haemoglobin variants include the  $\alpha$ - and  $\beta$ -Thalassaemia syndromes. These are inherited mutations, characterised by down regulation of globin gene expression resulting in total absence or quantitative reduction in production of either  $\alpha$  (in the case of  $\alpha$ -thalassaemias) or  $\beta$  (in the case of  $\beta$ -thalassaemias and HbE) globin chains (Steinberg, *et al* 2001). There is

great diversity in the type of mutations which result in the thalassaemia syndromes. The  $\beta$ -thalassaemia syndromes result mainly from point mutations affecting the  $\beta$ -globin gene itself; other mutations include deletions of the  $\beta$ -globin locus of varying sizes and mutations at distant locations perhaps affecting transcription factors. Point mutations down-regulate the  $\beta$ -globin gene by affecting the promoter regions, or causing premature termination through splice sites, nonsense mutations and frame-shifts. Similarly, the range of mutations causing  $\alpha$ -thalassaemia are equally diverse, although the majority, in contrast to those causing  $\beta$ -thalassaemia are deletions of the  $\alpha$ -globin genes. The clinical features of the thalassaemias are caused by anaemia (due to a reduced Hb) as well as the degree of imbalance between the  $\alpha$ - and non  $\alpha$ -globin chains.

HbE results from a point mutation ( $\beta^{26}\text{Glu}\rightarrow\text{Lys}$ ).  $\beta^E$  is also synthesised at a reduced rate compared with  $\beta^A$  and therefore has a thalassaemic phenotype. It is highly prevalent in South Asia where carrier frequency approaches 40% in some regions. Because of its thalassaemic features, if co-inherited with a  $\beta$ -thalassaemia causing mutation the combination can result in severe transfusion-dependant anaemia.

Qualitative variants result in production of structurally abnormal haemoglobin, some of which can also be reduced in quantity, e.g. HbE. The majority of Hb variants do not cause significant pathological effects, however mutations resulting in a change in charge and solubility (HbS  $\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Val}}$ , Hb C  $\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Lys}}$ ) or oxygen affinity (Hb Chesapeake  $\alpha_2^{92\text{Arg}\rightarrow\text{Leu}}\beta_2$ ) can cause clinically significant changes in function (Huisman, *et al* 1996). The significant qualitative variants are outlined in Table 1 below.

### 1.1.3 Haemoglobin S

Haemoglobin S (HbS) is caused by a mutation in the  $\beta$ -globin gene in which the 17<sup>th</sup> nucleotide is changed from thymine to adenine and thus the sixth amino acid in the  $\beta$ -globin chain becomes valine (neutral charge) instead of glutamic acid (negative charge). In the deoxygenated state (when conformational change causes the valine to be exposed) this leads to polymerisation and deformation of the red blood cell (RBC) into a “sickle” shape. Polymerisation of HbS is critically dependant on the intracellular HbS concentration. Sickle carriers with a HbS concentration of 35-40% (the majority of the rest being HbA) are asymptomatic except under extreme conditions (high altitude, extreme exercise). In this

respect, clinically, sickle cell disease is considered as a Mendelian recessive condition but haematologically as dominant. The homozygous inheritance of HbS or co-inheritance with particular haemoglobin variants results in a clinically significant haemoglobinopathy, the most common genotypes being listed in Table 1. Hb SS is the most common genotype, followed by Hb SC and Hb S $\beta$  thalassaemia. Hb SE is becoming increasingly common, particularly in the USA due to population migration from the Far East and intermarriage. In patients with African heritage HbSS makes up 70% of cases of SCD, with the majority of the remainder having HbSC (co-inheritance of  $\beta^S$  and  $\beta^C$  alleles) (Modell 2008).

Table 1: Significant sickle cell diseases in the UK

	Characteristics
<b>Severe sickle cell disease</b>	
Hb SS	The most common form of sickle cell disease
Hb S $\beta^0$ thalassaemia	Most commonly found in the eastern Mediterranean region and India
<b>Moderate sickle cell disease</b>	
Hb SC	25-30% of cases of SCD in populations of African origin
Hb S $\beta^+$ thalassaemia	Most cases found in the eastern Mediterranean region
<b>Mild sickle cell disease</b>	
Hb S $\beta^{++}$ thalassaemia	Mostly in African populations
<b>Very mild sickle cell disease</b>	
Hb SHPFH	Group of disorders caused by co-inheritance of HbS and HPFH

## 1.2 Sickle Cell Disease: Pathophysiology

The polymerisation of HbS is the basis of all sickle-related pathologies. In the deoxygenated state the abnormal valine is exposed on the outside of the haemoglobin tetramer resulting in a hydrophobic bond between the beta chains of two haemoglobin molecules (Brittenham, *et al* 1985). If this persists, the polymerised chains increase in size within the red blood cell reducing its flexibility and distorting its shape into the classical sickle form. The rate of polymerisation and therefore the type of distortion which affects the red cell is dependent on a) the degree of oxygenation of the cell b) intracellular pH and c) the intracellular HbS concentration (Bunn 1997). The consequences of polymerisation are subdivided into two major processes: vaso-occlusion and haemolytic anaemia (see

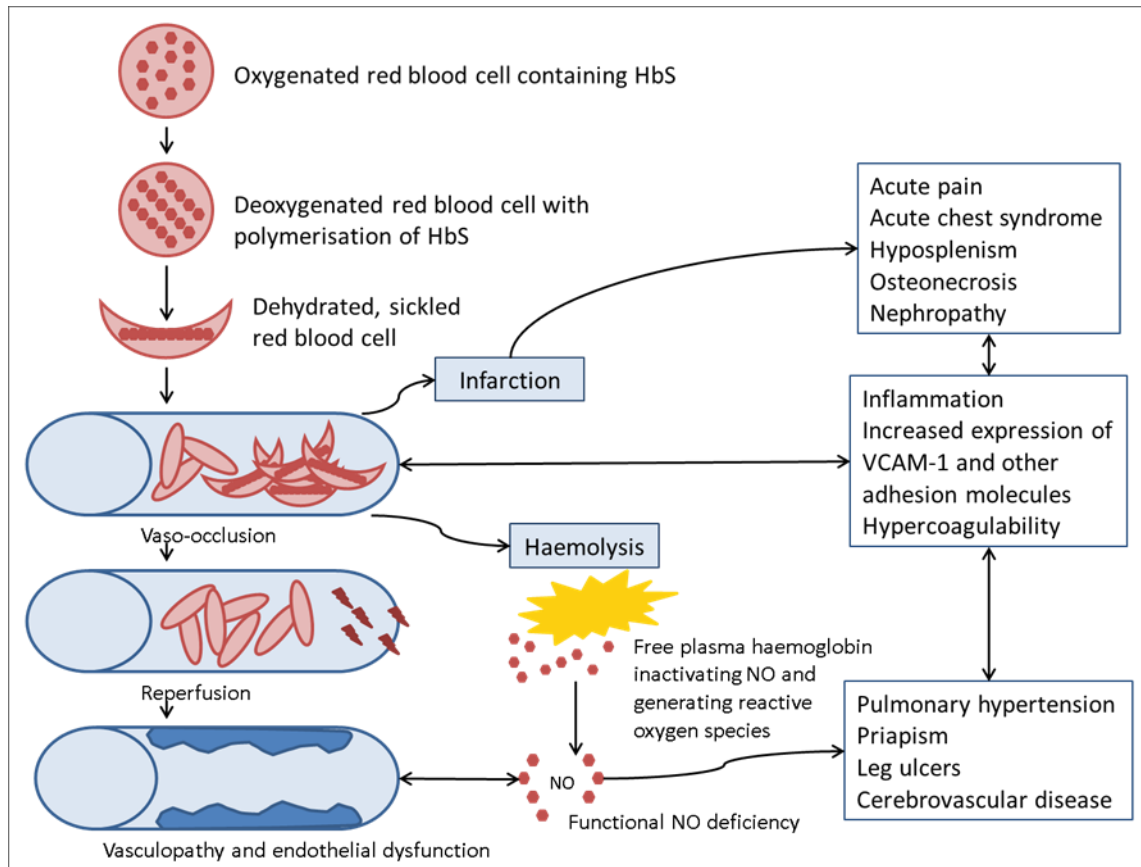


Figure 1) both of which result in vasculopathy and endothelial dysfunction.



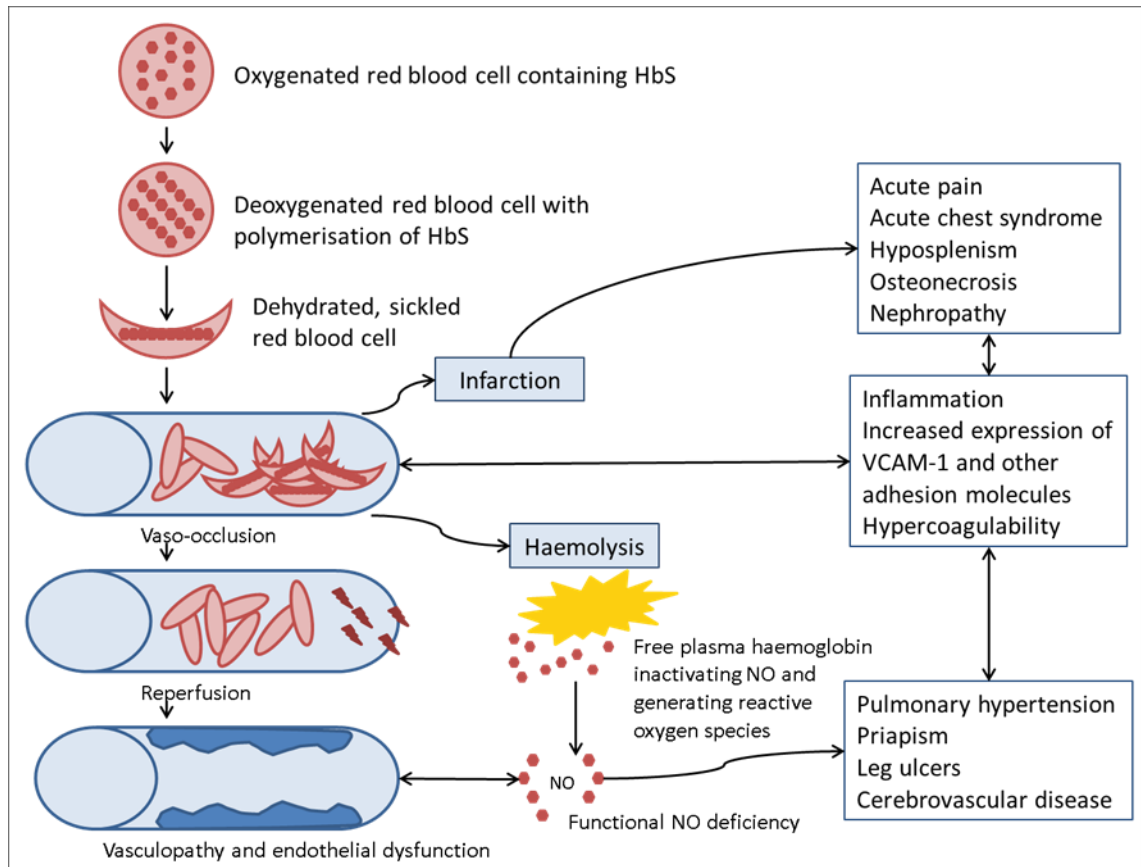


Figure 1: Pathophysiology of sickle-cell disease (Rees, *et al* 2010) Adapted from Rees *et al* 2010 with permission

Haemolysis and vaso-occlusion are thought to be at opposite ends of the pathological spectrum and patients are often described as having a “vaso-occlusive” or “haemolytic” phenotype depending on their presentation.

Haemolysis results from sickled cells being prematurely removed from the circulation; the red blood cell life span is significantly reduced (17 days vs. 120 days for normal controls) (Sydenstricker 1924). Patients with more prominent haemolysis trend towards a lower Hb, higher lactate dehydrogenase (LDH), higher bilirubin. Associations have been found with complications such as priapism and leg ulcers (Kato, *et al* 2007, Rees, *et al* 2010). The haemolysis which occurs in SCD is intravascular, potentially resulting in an effect on the bioavailability of nitric oxide (NO) which is postulated to be the causative mechanism behind the development of these complications. Nitric oxide controls vasodilation via binding of soluble guanylate cyclase, leading to the conversion of GTP to cGMP, which in turn relaxes vascular

smooth muscle causing vasodilatation. Plasma Hb released during intravascular haemolysis leads to NO consumption, producing methemoglobin and bio-inactive nitrate. A state of reduced endothelial NO bioavailability therefore exists in SCD causing impairment of downstream homeostatic vascular functions of NO, including inhibition of platelet activation and aggregation and reducing expression of the cell adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), P-selectin and E-selectin. Haemolysed RBCs also release arginase which breaks down the L-arginine which is a substrate for NO production, further reducing endothelial NO. Thus SCD patients are predisposed to vasoconstriction, endothelial activation and proliferation. Clinical complications linked to the haemolytic phenotype include leg ulcers, priapism and pulmonary hypertension.

Vaso-occlusion has the polymerisation of HbS at its heart. Direct interaction between the sickled erythrocytes/reticulocytes and the endothelial cell wall of blood vessels causes interruption of normal tissue perfusion to down-stream tissues, leading to decreased oxygenation, further increasing sickling. The white blood cell count (WBC) and adhesion molecules on the surface of the endothelium contribute to the adherence of erythrocytes; the expression of the later is controlled by NO, as explained above. Patients with the vaso-occlusive phenotype have higher Hb, lower LDH and bilirubin but increased frequency of acute pain. This phenotype is associated with complications related to micro-vascular occlusion such as acute chest syndrome (ACS) and avascular necrosis (AVN).

Various therapeutic strategies have been designed to alter these stages which are discussed further in section 1.5.

### **1.3 Phenotypic Diversity**

SCD appears to be a simple Mendelian autosomal recessive disorder. Yet, the range of complications and variability in biological parameters is large and any organ can be affected. Patients may develop all or none of the complications listed below in a variety of different combinations although certain complications are associated with either the vaso-occlusive or haemolytic phenotypes and therefore are more likely to occur together. The same diversity is found in patients' responses to treatments such as hydroxycarbamide (hydroxyurea, HC) which has a response rate of approximately 70%.

### **1.3.1 Clinical Manifestations of Sickle Cell Disease**

Fifty years ago SCD was largely a disease of childhood, although various groups reported small groups of patients surviving into their fourth decade. Differences in severity between the different sickle genotypes are well documented; Hb SC is a more benign condition than Hb SS (although with increased risk of specific complications), Hb S $\beta^0$  of equivalent severity to Hb SS and Hb S $\beta^+$  of milder severity due to the reduced concentration of HbS.

Hyposplenism is one of the earliest causes of morbidity and mortality in SCD with almost 90% of children recruited to the BABY HUG trial having reduced (75%) or absent (15%) splenic function by the age of 18 months (Wang, *et al* 2011). It is unsurprising therefore that bacterial infections (particularly those secondary to capsulated pathogens) are a major cause of morbidity and mortality in SCD, prior to the introduction of penicillin prophylaxis (Gaston, *et al* 1986, Williams, *et al* 2009). The increased susceptibility is likely to be caused by impaired splenic function (secondary to vaso-occlusion in the splenic vascular bed), defects in complement activation and tissue ischaemia. The introduction of penicillin prophylaxis and immunisation against the capsulated organisms *S. pneumonia* and *H. influenza* has caused substantial improvements in prognosis (Halasa, *et al* 2007).

#### **1.3.1.1 Pain**

Pain is the most common presentation of SCD and is the result of tissue ischemia secondary to vaso-occlusion. It is extremely variable in location, severity, duration and causes of exacerbation (Ballas and Lusardi 2005, Platt, *et al* 1991). Patients who have higher Hb levels have a more vaso-occlusive phenotype and appear to be at increased risk of acute painful episodes. Cultural and psychological influences may affect patients' interpretation of pain. Recurrent acute painful episodes can lead to long-term tissue damage and therefore chronic pain. Prompt treatment with appropriate analgesics and individualised care plans are used to tailor treatment on a patient-by-patient basis (Rees, *et al* 2003).

#### **1.3.1.2 Anaemia**

Chronic anaemia, secondary to haemolysis, is a hallmark of SCD, although severity varies across and even within the sickle genotypes. The severity of the anaemia can increase acutely

due to a variety of causes. An acute increase in haemolysis secondary to acute polymerisation can be triggered by a variety of causes including infection, cold or decreased oxygen tension. This is usually accompanied by an episode of pain. Red blood cells can also become acutely trapped in the liver and spleen (also known as sequestration). This results in an exacerbation of anaemia presenting with hepato- or splenomegaly, hypovolaemia and shock. It usually occurs in childhood (Emond, *et al* 1985, Solanki, *et al* 1986). A reduction or total suppression of red blood cell production can occur in the bone marrow resulting in a transient but profound anaemia. This classically occurs due to parvovirus B19 (which is directly toxic to erythroid precursors in the bone marrow) but can be due to other infections. Treatment can also cause bone marrow suppression and this is carefully monitored for in HC treatment. When anaemia of any cause becomes symptomatic, transfusion may be required.

#### **1.3.1.3 Neurological**

Stroke is one of the most devastating consequences of SCD and present acutely (as overt stroke or transient ischaemic attack) or be “silent”, discovered only on imaging of the brain. Overt stroke occurs in about 11% of patients with SCD by the age of 20 years (Ohene-Frempong, *et al* 1998, Powars, *et al* 1978). It can be ischaemic, secondary to arterial stenosis caused by sickle vasculopathy (most commonly of the internal carotid and middle cerebral arteries) or haemorrhagic.

Ischaemic strokes are most frequently found in children aged from 2 to 9 years, with a peak around the 2 to 5 age group and present with unilateral weakness, facial drooping and sometimes aphasia. They can be difficult to diagnose, particularly in younger children with limited communication skills, and are sometimes misdiagnosed as painful episodes as the child appears to be not using a limb. Overt strokes are treated with exchange transfusion with the aim of reducing the HbS to less than 30%. Transfusion is also used for ongoing secondary prevention (Lusher, *et al* 1976) in these patients. Key trials have shown that raised transcranial doppler (TCD) velocities in the middle cerebral or carotid arteries are significantly associated with increased risk of stroke (Adams, *et al* 1992, Adams, *et al* 1998) and that blood transfusion is efficacious in primary stroke prevention (the stroke prevention trial in sickle cell anaemia [STOP] and STOP2 studies), (Adams and Brambilla 2005, Adams, *et al* 1998). This has led to

a proactive annual screening programme in the UK for all children aged from 2 to 18 years old in the UK using TCD velocities. Those with abnormal velocities then progress to further imaging and are commenced on a transfusion programme.

Patients can also present with transient ischaemic attacks (TIAs) which have similar neurological features to an overt stroke but resolve within 24 hours. In the majority of cases abnormal imaging will be found resulting in the episode being treated in the same way as an established stroke.

As the sickle cell population ages, more “traditional”, non-sickle related ischaemic strokes can occur secondary to more standard risk factors including age, smoking and hypertension. There is currently no agreement on whether these patients should be treated with anti-platelet agents or whether that puts them at risk of haemorrhagic stroke due to underlying sickle vasculopathy affecting cerebral vessels.

Intense headache is a more prominent feature of haemorrhagic strokes accompanied by variable degrees of weakness. They occur as the result of underlying chronic damage to cerebral vessels and subsequent neo-vascularisation with fragile collaterals, described as “Moya-Moya” due to their smoke like appearance on imaging (Stockman, *et al* 1972). Patients with a previous history of ischaemic stroke in childhood can present with haemorrhagic stroke as adults. The peak occurrence is in older adults (aged 20-29 years) although haemorrhage can occur in any age group. Patients are treated using transfusion to reduce the S% and prevent progression of neo-vascularisation.

There is also a role for neurosurgery, both acutely to treat complications from the haemorrhage and also in the chronic setting to improve blood flow to the brain and reduce neovascularisation.

Silent infarcts are also found in SCD, being seen only on imaging (Switzer, *et al* 2006), although retrospective analysis can sometimes show gradual deterioration in physical function or academic performance. Approximately 25% of children with SCA will have a silent infarct by their 6<sup>th</sup> birthday (Kwiatkowski, *et al* 2009) and approximately 33% before their 13<sup>th</sup> birthday (DeBaun, *et al* 2012). The role of treatment in silent infarct is controversial and a clinical trial is ongoing to determine the efficacy of transfusion in the prevention and limiting progression of silent infarcts (NCT00072761).

#### 1.3.1.4 Cardiac and Respiratory

Chronic anaemia and the resulting high cardiac output state eventually causes hypertrophy of the left ventricle and septum leading to left ventricular dysfunction in both systole and diastole. This is usually well compensated by increased cardiac pre-load and decreased after-load, however congestive cardiac failure may result when these mechanisms fail. Diastolic dysfunction is associated with increased mortality (Sachdev, *et al* 2007).

Lung complications can be acute or chronic. Acute chest syndrome (ACS) is the second most common reason for hospital admission in SCD, although 50% occur on the background of a pre-existing painful vaso-occlusive episode. ACS is caused by vaso-occlusion of pulmonary vasculature causing respiratory distress syndrome and can be triggered by a combination of infection and fat embolism resulting in acute lung injury. 13% require mechanical ventilation and death occurs in 3% of patients (in a mixed adult and paediatric study) (Vichinsky, *et al* 2000). The clinical features of ACS are reduced oxygen saturations or new pulmonary infiltrates on X-ray. Low oxygen saturations, increased respiratory rate and breathlessness may also be seen. Non sickle risk factors for ACS include underlying asthma (Boyd, *et al* 2006, Boyd, *et al* 2007) which is also associated with overall mortality in SCD.

Chronic respiratory complications include chronic sickle lung disease (SCLD) and pulmonary hypertension (PHT). Chronic sickle lung disease has a prevalence of approximately 4% (Powars, *et al* 1988). It is thought to be related to recurrent vaso-occlusive and infective events in the lungs and is characterised by a restrictive airways defect and interstitial lung abnormalities (Powars, *et al* 1988).

Transthoracic echocardiogram is a commonly used screening tool for PHT, however having a raised TRJet velocity does not necessarily mean that a patient has true pulmonary hypertension. In SCD there are a variety of potential causes of this; 1) chronic anaemia leads to a hyperdynamic circulation resulting in left ventricular remodelling and diastolic dysfunction, 2) repeated vaso-occlusive events in the lungs causing parenchymal damage and “back pressure” on the pulmonary system, 3) chronic thromboembolic disease resulting in increased resistance in the pulmonary vasculature and 4) “true” pulmonary hypertension secondary to increased pulmonary vascular resistance from vasculopathy associated with haemolysis.

In 2004 Gladwin *et al* used tricuspid regurgitant jet (TRJet) velocity to diagnose PHT. They found 32% of SCD patients had raised estimated pulmonary artery pressures (>35mm Hg) and 9% moderate to severe pulmonary hypertension (>45 mm Hg) and that this was associated with increased mortality (Gladwin, *et al* 2004). There is now evidence that this was an over estimation; a French study using cardiac catheterisation found a prevalence of 6% (Parent, *et al* 2011), and a re-analysis of the American cohort and data from the Brazilian cohort confirm the prevalence at 6-7% (Fonseca, *et al* 2012). Nonetheless, a raised TRJet velocity is a marker of sickle mortality.

#### **1.3.1.5 Renal**

Renal damage is common in SCD due to the underlying environment of the renal medulla; where low partial pressure of oxygen, low pH and high osmolality combine to create optimum conditions for HbS polymerisation and sickling (Sharpe and Thein 2011). This leads to recurrent vaso-occlusion and chronic ischaemia resulting in papillary necrosis and medullary fibrosis (focal segmental glomerulosclerosis). This can present in the acute situation as haematuria. Chronically this results in glomerular hyperfiltration (seen in childhood and early adulthood) and protein loss in the urine (Becton, *et al* 2010, Scheinman 2009), an early sign of which is micro-albuminuria. The raised glomerular filtration rate (GFR) appears to be mediated by prostaglandins (Scheinman 2009). Some patients appear to move rapidly to decreased GFR and resulting end-stage renal failure (Ataga 2009) although the reasons for this are unknown. (See Chapter 6).

#### **1.3.1.6 Priapism**

Priapism is characterised as an unwanted, painful and persistent erection of the penis. It is caused by either increased arterial inflow (i.e. high flow) or, more commonly, the failure of venous outflow (i.e. low flow), resulting in blood trapping within the erectile bodies. Approximately 30% of males with SCD under the age of 20 years reported having had at least one episode of priapism, whereas frequencies of 30% to 45% are estimated for adult men (Bruno, *et al* 2001, Fowler, *et al* 1991). Episodes can be stuttering (lasting less than 4 hours) or prolonged (greater than four hours); the later carrying the risk of ischaemia, fibrosis and loss of erectile function. Priapism is associated with the haemolytic phenotype (Nolan, *et al* 2005b).

#### **1.3.1.7 Bone**

Avascular necrosis (AVN) occurs due to infarction of the articular surfaces of the long bones secondary to vaso-occlusion. The prevalence of AVN varies according to the sensitivity of the diagnostic method; using X-ray Milner *et al* found evidence of femoral AVN in 9.8% of patients (Milner, *et al* 1991) compared to Ware *et al* who found prevalence of 41% using MRI (Ware, *et al* 1991). The most common sites of osteonecrosis are the femoral heads, followed by the head of the humerus. Various interventions are possible, from total replacement of the joint in advanced disease with collapse of the femoral head, to core decompression in early disease (Neumayr, *et al* 2006). AVN is associated with the vaso-occlusive phenotype and is almost as prevalent in Hb SC disease as it is in Hb SS.

#### **1.3.1.8 Liver**

The descriptor “sickle cell hepatopathy” covers both acute and chronic manifestations of liver disease in sickle cell patients. The acute presentations are extremely varied. Gallstones are common in patients with SCD and this can result in an acute transaminase rise during episodes of acute cholecystitis, usually accompanied by right upper quadrant pain and sometimes fever. “Hepatic crisis” with intra-hepatic cholangiography can accompany generalised vaso-occlusive pain and is secondary to acute vaso-occlusive events in the liver. Hepatic sequestration is one of the most dramatic liver complications of SCD, during which large volumes of blood are trapped in the liver resulting in anaemia and hypovolaemia although the liver dysfunction may be mild. The diagnosis consists of a significant decrease in Hb from baseline with associated liver enlargement. During “hepatic crises” the vaso-occlusion causes anoxia and ballooning of hepatocytes that leads to intrahepatic cholestasis (Ballas, *et al* 2010, Banerjee, *et al* 2001, Berry, *et al* 2007).

The chronic presentations of liver disease in SCD are less clearly defined and indeed as liver disease is largely asymptomatic until significantly advanced, the true incidence of liver involvement in sickle cell disease is unknown (see Chapter 7).

#### **1.3.1.9 Eye**

Numerous retinal changes have been described in SCD. Angioid streaks (cracks in Bruch’s membrane characterised by calcium deposition (Nagpal, *et al* 1976)), black sunbursts (lesions in the retina which form at the border of perfused and non-perfused retina (Asdourian, *et al*



1975)) and salmon patch haemorrhages (small intra retinal haematomas (Gagliano and Goldberg 1989)). These are all the result of proliferative sickle retinopathy which has similar pathophysiology to other neo-vascularisation disorders of the retina. Vaso-occlusion of peripheral arterioles leads to retinal ischaemia. This in turn leads to secretion of vascular growth factors and formation of new vessels at the interface of perfused and non-perfused retina that are highly fragile and predisposed to haemorrhage, although they may auto infarct (Condon, *et al* 1980, Condon and Serjeant 1980). Vitrectomy may be required if haemorrhage does not spontaneously resolve. Severe sickle retinopathy can result in retinal detachment and loss of vision.

### **1.3.2 Definitions of Severity**

The definition of severity within the sickle genotypes has largely depended on the development of scoring systems which include the various complications; here the diversity of SCD and its clinical complications renders the process difficult. However defining “severe disease” is important to performing genetic association studies, targeting and assessing response to treatments and assessing the impact of other factors such as age, gender and social effects.

Historically, scores have been based around number of clinical presentations with vaso-occlusive crisis per patient year, evidence of end-organ damage, transfusion requirements and functional status (employment) (Serjeant 1975, Steinberg, *et al* 1973). There are a number of potential problems with this approach; 1) pain is extremely subjective and patients have different thresholds for presenting to hospital that can be influenced by a large range of biological and social variables, 2) definitions of end-organ damage are not standardised, leading to issues comparing across data sets, although findings are more objective, 3) the frequency of measurable organ damage can be low within a population, making genetic studies difficult.

Previous scores have been weighted to give increased importance to complications regarded as more significant such as cerebrovascular disease or acute chest syndrome (Afenyi-Annan, *et al* 2008). More recently clinical data has been combined with rheological results to attempt to predict the development of severe disease and mortality using Bayesian networks (Sebastiani, *et al* 2007).

Recently a consensus has been attempted with regard to defining end-organ damage to enable international standardisation and comparison of results from study to study (Ballas, *et al* 2010).

## **1.4 Predictors of Severity**

As discussed above the definition of severe disease in SCD has been a matter for debate. As health provision comes increasingly under the public and political gaze, the focus has switched from reactive medicine (dealing with complications as and when they arise) to preventative and predictive medicine. The accurate definition of predictors of severity could enable more targeted treatments, the early treatment or prevention of complications and hopefully, an overall reduction in rates of morbidity and mortality.

### **1.4.1 Genetic Predictors of Severity**

Increasingly technological advances have enabled new insights to be gained into the underlying genetic influences on disease severity. Accurate, high throughput genotyping and sequencing have allowed links to be drawn between clinical outcomes and complications and the inheritance of specific genetic polymorphisms. The genetic background on which the sickle mutation occurs is thought to be an example of this genetic modulation.

### **1.4.2 Fetal Haemoglobin**

Fetal haemoglobin, made up of  $\alpha_2\gamma_2$  sub-units, represents 90–95% of all haemoglobin by 34-36 weeks gestation. Adult haemoglobin, HbA, ( $\alpha_2\beta_2$ ) accounts for 4–13% of total haemoglobin at this stage. After 34 weeks gestation, HbA production increases significantly as HbF production begins to fall. By 40 weeks, HbF represents 53–80% of all haemoglobin with HbA levels reaching 20–30% at the time of delivery. HbF is normally less than 2–3% of total haemoglobin by 6 months of age (see Figure 2).

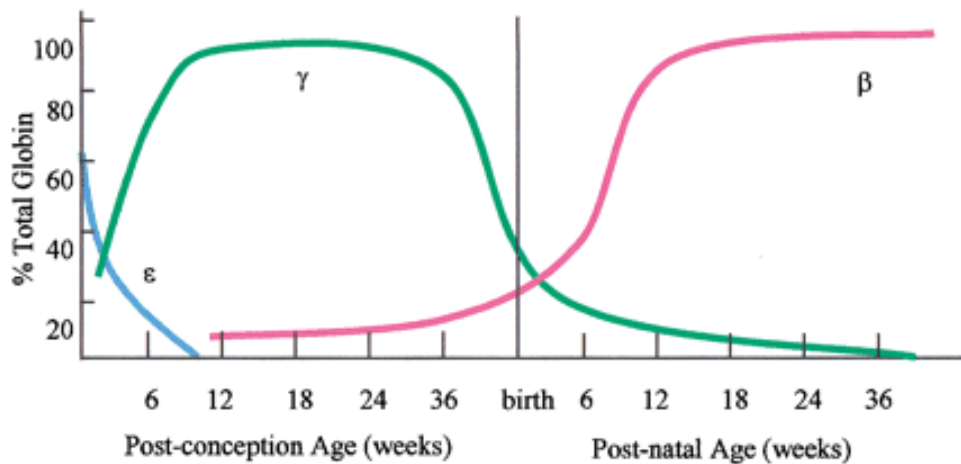


Figure 2:  $\beta$ -like globin gene expression.

The embryonic gene is expressed during the first six weeks of gestation. The first switch from  $\epsilon$ - to  $\gamma$ -globin occurs shortly after conception, and the second switch from  $\gamma$ - to  $\beta$ -globin occurs shortly after birth (Harju, *et al* 2002)

HbF falls to less than 0.6% of the total haemoglobin during adulthood, however higher levels persist in some individuals. Although this raised level of HbF gives no advantage or disadvantage in healthy individuals, it is an important ameliorating factor in SCD, an inverse relationship between HbF levels and early mortality have been noted (Platt, *et al* 1994, Powars, *et al* 1984). There are two main reasons for this effect. 1) The presence of HbF inhibits HbS polymerisation and 2) its presence reduces the concentration of HbS in the red cell (Noguchi, *et al* 1993). Persistence of very high levels of HbF (levels of 10-40%) is most commonly due to deletions in the *HBB* cluster or point mutations in the  $\gamma$  promoter. These individuals are grouped under the heading of Hb S/HPFH, the HPFH is also inherited in a Mendelian manner. However these HPFH mutations are rare and do not explain the common variations of HbF found in the SCD population, which are related to three major genes/genetic regions also referred to as quantitative trait loci (QTLs). These include a QTL in the  $\beta$ -globin gene cluster on chromosome 11 tagged by the *Xmn1*-G $\gamma$  (C/T) variant, the *HBS1L-MYB* intergenic region on chromosome 6q23 and the *BCL11A* gene on chromosome 2p15 (Menzel, *et al* 2007, Thein and Menzel 2009, Thein, *et al* 2007). The three HbF QTLs account for up to 20% of HbF variation in African-American patients with SCD with a corresponding reduction in acute pain (Lettre, *et al* 2008). The association between HbF levels and improved outcomes has led to the development of

different approaches to HbF augmentation, the main pharmacological treatment being HC (see 1.5.2.1).

### **1.4.3 Alpha-thalassaemia**

In patients of African descent, co-inheritance of  $\alpha$ -thalassaemia is common: approximately 30% are carriers for a single  $\alpha$ -globin gene deletion ( $\alpha\alpha/-\alpha$ ), and a further 5% are homozygous ( $-\alpha/-\alpha$ ) (Steinberg, *et al* 1986, Steinberg, *et al* 2001, Vasavda, *et al* 2007). Co-inherited  $\alpha$  thalassaemia in SCD ( $\alpha$ -SCD) impacts on the haematological indices and erythrocyte rheology; the reduced intracellular HbS (due to reduced availability of alpha-chains) reduces the threshold for Hb S polymerisation, equating to reduced sickling, a lower rate of haemolysis and higher hematocrit with a corresponding increase in blood viscosity, resulting in increased vaso-occlusive events and relatively more acute painful episodes (Ballas 2001). Patients with  $\alpha$ -SCD appear to be protected against complications associated with the haemolytic phenotype such as leg ulcers, PHT, priapism and albuminuria (Higgs, *et al* 1982, Nolan, *et al* 2005b, Steinberg, *et al* 1984).  $\alpha$ -SCD also appears to blunt the response to HC in patients with SCD (Vasavda, *et al* 2008).

### **1.4.4 Other Polymorphisms Associated with Specific Complications**

#### **1.4.4.1 Stroke**

In the general population ischaemic stroke is thought to be the result of multiple gene and environmental interactions, with no single predisposing polymorphism (Matarin, *et al* 2007). In SCD a variety of genes/SNPs have been associated with stroke, and studies have focused on looking at candidate genes in combination (Hoppe, *et al* 2004) and Human leukocyte antigen (HLA) genotypes (Hoppe, *et al* 2003). Some of the associated genes/SNPs are associated with cell adhesion and inflammation (known to be part of the pathogenesis of stroke). These include *VCAM1*, *IL4R*, *TNFA*, *SELP*. *VCAM1* and *TNFA* all of which have been implicated in several studies (Hoppe, *et al* 2004, Hoppe, *et al* 2007, Sebastiani, *et al* 2005).

#### **1.4.4.2 Bilirubin Levels and Cholelithiasis**

Gallstones are a common complication in children with SCD with a reported frequency of up to 12% as early as 2 to 4 years of age (Sarnaik, *et al* 1980). Prevalence of 30%-70% in all age groups has been reported in the United States (Barrett-Connor 1968, Sarnaik, *et al* 1980). The majority of gallstones in SCD patients are pigment stones, in comparison of approximately 15%

in the general population. An increased number of TA repeats in the promoter region of uridine diphosphoglucuronate glucuronosyltransferase 1A (*UGT1A1*) has consistently been associated with raised bilirubin levels and therefore of risk of development of pigment gallstones due to inefficient bilirubin conjugation (Chaar, *et al* 2006a, Passon, *et al* 2001, Vasavda, *et al* 2007).

#### **1.4.4.3 Avascular Necrosis**

Development of AVN has been associated with the vaso-occlusive phenotype and Hb SC disease. The *MTHFR* gene has been associated with the development of AVN in both the American and Brazilian SCD populations (Andrade, *et al* 1998, Kutlar, *et al* 2001). Baldwin *et al* compared frequency of candidate genes with controls and found significant associations with SNPs in *BMP6*, *TGFB2*, *TGFB2*, *KL*, and *ANXA2* genes (Baldwin, *et al* 2005). Our own group replicated the association with *BMP6* (Ulug, *et al* 2009). The precise mechanism of how these polymorphisms could contribute to the development of AVN is currently unclear.

#### **1.4.4.4 Acute Chest Syndrome**

Polymorphisms in the genes coding for endothelial nitric oxide synthase (eNOS3) have been associated with the development of ACS in females (Sharan, *et al* 2004). In a separate study exhaled NO levels were reduced in ACS compared to controls, and this was associated with the number of AAT repeats in intron 20 of *NOS1* (Chaar, *et al* 2006b). Reduced levels of heme oxygenase may result in reduced nitric oxide availability, endothelial dysfunction and vasculopathy, all implicated in the pathogenesis of acute chest syndrome. Polymorphisms in the *HMOX-1* gene (the number of repeats in the promoter) have been found to influence admissions for ACS, shorter alleles ( $\leq 25$  repeats) associated with lower rates of hospitalisation with acute chest syndrome (Bean, *et al* 2012).

#### **1.4.4.5 Leg Ulcers**

Leg ulcers are strongly associated with the severity of haemolysis. Nolan *et al* found that SNPs in *KLOTHO* (a gene with a role in endothelium-derived NO production and thus oxidative biology), *TEK* (an endothelial-expressed gene involved in endothelial smooth muscle cell communication) and genes in the *TGF- $\beta$ /BMP/SMAD* signalling pathway (involved in cellular differentiation, apoptosis and cellular homeostasis) were associated with leg ulcers (Nolan, *et al* 2006). There are also geographical variations in prevalence of leg ulcers, a complication found

in up to 70% of Jamaican Hb SS patients with a lower incidence of 30-40% in other populations (Alexander, *et al* 2004).

#### **1.4.4.6 Priapism**

Priapism is another complication of SCD which has been linked to haemolysis. Again polymorphisms in the *KLOTHO* and also genes in the *TGF- $\beta$ /BMP/SMAD* pathway have been associated with the development of priapism (Elliott, *et al* 2007, Nolan, *et al* 2005a, Nolan, *et al* 2005b). Klotho-deficient mice exhibit impaired endothelium dependent vasodilatation and impaired angiogenesis, suggesting that Klotho protein may protect the cardiovascular system through endothelium-derived NO production.

#### **1.4.4.7 Renal Dysfunction**

Genes in the *TGF- $\beta$ /BMP/SMAD* pathway have also been associated with the development of sickle cell nephropathy, using eGFR as a marker of renal function (Nolan, *et al* 2007). As with other complications associated with the haemolytic phenotype, co-inheritance of  $\alpha$ -thalassaemia appears to offer protection against microalbuminuria (Day, *et al* 2012). Polymorphisms in the *MYH9* and *APOL1* genes have been linked to development of FSGS (Ashley-Koch, *et al* 2011, Freedman, *et al* 2010, Genovese, *et al* 2010a, Genovese, *et al* 2010b, Tzur, *et al* 2010).

## **1.5 Treatment Options**

Treatments for sickle cell can broadly be divided into supportive, disease modifying and organ specific therapies.

### **1.5.1 Supportive**

#### **1.5.1.1 Analgesia**

As discussed in 1.3.1.1 pain is the most common cause of presentation to hospital. Prompt treatment with appropriate analgesia is essential and patients should have their own individualised pain plans. These are based around the analgesic ladder, commencing with simple analgesia and escalating to opiates, sometimes delivered via patient controlled systems (Rees, *et al* 2003). Opiate toxicity has been highlighted as an action point by the recent National Confidential Enquiry into Patient Outcomes and Death (NCEPOD) report (Lucas 2008).

### 1.5.1.2 Blood Transfusion

Transfusion of normal red blood cells provides benefit by correcting the low-oxygen capacity caused by the anaemia as well as improving microvascular perfusion by reducing the proportion of sickle red cells in circulation. Regular blood transfusion also suppresses endogenous erythropoiesis and the production of sickle haemoglobin.

Although the clinical benefits of blood transfusion for patients with SCD have long been recognised, complications associated with transfusion have limited its use. These include the development of alloimmunisation, fears about infection being transmitted via blood products and iron-overload. There is a strong evidence base for using transfusion in primary (Adams and Brambilla 2005, Adams, *et al* 1998) and secondary prevention of stroke (Lusher, *et al* 1976) and in the treatment of ACS (Styles, *et al* 2007, Vichinsky, *et al* 2000). Transfusion Preoperatively in Sickle Cell Disease (TAPS - National clinical trial number: [NCT:] 00512577) recently showed improved outcomes in patients who received transfusion or exchange for medium risk surgical procedures (Howard, *et al* 2011).

Blood transfusion can be a simple top-up of additional units of blood without removal of sickle blood, or exchange (automated or manual) with the removal of sickle blood that is replaced by normal red cells (Wahl and Quirolo 2009). Simple top-up transfusions are easy to administer and minimise donor exposure, however long term can lead to significant accrual of iron. However caution should be exercised in patients with higher haemoglobin levels, with the target haemoglobin usually set around 100g/L for those patients having episodic transfusions due to concerns about hyperviscosity. Exchange transfusion requires specially trained staff, and in the case of automated exchange, equipment. Good intravenous access is also required to get sufficient flow rates across the apheresis machine. Due to the larger volumes of blood used (8-12 units compared to 1-3 units for simple top-up) donor exposure is increased leading to increased risk of sensitisation (Wahl and Quirolo 2009). However, with no net increase in blood transfused, the risk of iron overload is significantly reduced, and in some cases, patients on regular exchange apheresis have been rendered iron deficient

Transfusions can be episodic or part of a chronic transfusion programme; whether the transfusion is a simple top-up or exchange is guided by the indication and clinical presentation.

Some indications for transfusion are controversial and are dependent on clinician and patient preference.

Strong indications for episodic transfusion include: 1) the treatment of acute anaemia, (acute splenic sequestration (a simple transfusion to reverse hypovolaemia and cardiovascular decompensation) and transient red cell aplasia (simple transfusion to reverse anaemia)); 2) the management of acute illness (acute chest syndrome (simple or exchange transfusion dependant on the initial haemoglobin and clinical status of the patient) and acute stroke (exchange transfusion to HbS level of less than 30%) and multi-organ failure (exchange transfusion therapy (to improve oxygenation of organs and improve survival)); and 3) preparation for general anaesthesia (as per the TAPS guidance). Other more controversial indications for episodic transfusion therapy include the management of acute priapism, pregnancy and transfusion prior to infusion of intravenous contrast media.

Strong indications for chronic transfusion include: 1) prevention of primary or secondary stroke (usually simple transfusion in children and exchange transfusion in adults); 2) post-organ transplant (usually exchange transfusion to preserve function of the graft); and 3) chronic sickle lung disease (usually simple top-up transfusion for symptomatic relief of breathlessness). Controversial indications include failure of hydroxycarbamide therapy (recurrent painful episodes or frequent hospital admissions), treatment of slow-healing leg ulcers and prevention of priapism. Once a transfusion programme is started it can be difficult to stop and therefore careful consideration should be undertaken prior to its initiation.

#### **1.5.1.3 Penicillin**

Used for prophylaxis against capsulated organisms (see 1.3.1).

### **1.5.2 Disease Modifying**

#### **1.5.2.1 Increasing HbF**

##### **Hydroxycarbamide**

Hydroxycarbamide (Hydroxyurea – HC) is a mitotic agent, first developed as chemotherapy, and is effective therapy in about 60-70% of patients with SCD. Main action is thought to be via increased HbF, partially explained by erythroid regeneration and recruitment of early progenitors, which produce higher levels of HbF (Dover, *et al* 1986). HC also increases mean



corpuscular volume thus lowering the intracellular concentration of HbS and reducing polymerisation. There are additional benefits to HC aside from increasing HbF levels; it lowers the white cell count, reticulocytes and platelets, increases nitrous oxide (NO) production (Nahavandi, *et al* 2002), improves red blood cell (RBC) hydration (Orringer, *et al* 1991), and reduces RBC adhesiveness to the endothelium (Hillery, *et al* 2000). By these mechanisms HC has been shown to reduce the number of painful crises and particularly the number of episodes of acute chest syndrome in a subsection of patients. Its effect on preservation of organ function (using the spleen as an example) was recently examined in the BABY HUG study (Wang, *et al* 2011).

HC is well tolerated, with few side effects. Reducing WBC is one of its therapeutic effects but can become an adverse event. The most significant is leucopenia, which can be severe. The dose is titrated according to patient response, the patient attending clinic regularly for full blood count (FBC) checks with the dose adjusted accordingly. The long term effects are still being studied in clinical trials (NCT: 00305175) and there are particular concerns about reduced fertility in male patients treated with HC (Brawley, *et al* 2008). This, coupled with patient concerns about the risk of carcinogenesis, mean HC is still currently underused.

### **Erythropoietin**

It has been shown that erythropoietin is able to increase HbF concentration when used in isolation in primates (Al-Khatti, *et al* 1987). Erythropoietin in combination with supplemental iron has produced an increase in HbF levels (Nagel, *et al* 1993, Rodgers, *et al* 1993). This response was durable despite erythropoietin being stopped so its mechanism of effect is unclear. A phase I trial (NCT: 00270478) combining HC with erythropoietin in patients with renal impairment or pulmonary hypertension completed in 2012 but unfortunately the results are not yet published.

### **Decitabine**

Decitabine (1-deoxy 5-azacytidine) is an analogue of 5-azacytidine, its mechanism of action is via hypomethylation of the HBG ( $\gamma$ -globin gene), increasing its expression and thus inducing  $\gamma$ -globin synthesis. This is referred to as the " $\gamma$ -globin reverse switch" and is the theoretical mechanism of action for increased HbF production (Charache, *et al* 1983). Decitabine has emerged as an alternative to 5-azacytidine due to concerns about its oncogenic potential,

despite initially promising results in SCD (Dover, *et al* 1985) (Carr, *et al* 1984). It appears to have a similar positive effect as 5-azacytidine while having no oncogenic problems (DeSimone, *et al* 2002). Decitabine has only been available in intravenous (IV) or sub-cutaneous (SC) forms although oral forms are currently in phase 1 studies (NCT: 01685515). A trial is currently recruiting (NCT: 01375608) to assess safety and efficacy in raising HbF levels in patients with SCD.

### **Butyrate and Histone Deacetylase (HDAC) Inhibitors**

Butyrates increase HbF levels by de-repressing  $\gamma$ -globin genes by increasing levels of core histone acetylation via inhibition of histone deacetylase, causing increased transcription rates of  $\gamma$ -globin genes (Weinberg, *et al* 2005). Its usefulness as a treatment is limited by its very short half-life and marrow toxicity. Oral analogues are available but very high doses are required to be effective and the tablets taste very unpleasant. Pulsed treatment appears to be effective leading to sustained improvements in HbF levels over several months (Atweh, *et al* 1999). A Phase II trial looking at the effects of arginine butyrate on healing times of leg ulcers showed positive results with significant improvement in healing over a 3 month period (McMahon, *et al* 2010). New short-chain fatty acid derivatives have been developed, however a recent study of HQK-1001 (2,2-dimethylbutyrate (HQK-1001), an oral fetal globin inducer synthesised by HemaQuest, Boston) was disappointing in terms of both HbF induction and anaemia (Reid, *et al* 2014). Clinical trials are ongoing in two other HDAC inhibitors which were identified in a library screen; a phase 1 study using pabinostat (NCT: 01245179) and a Phase 2 study examining the effect of vorinostat on HbF induction (NCT: 01000155); recent *in vitro* work has also favourably compared givinostat to hydroxycarbamide and butyrate (Ronzoni, *et al* 2014).

### **Thalidomide Derivatives**

Thalidomide and its derivatives Lenolidamide and Pomalidomide have been used to improve anaemia in selected patients with myelodysplastic syndromes and are the subject of multiple NIH trials. It has also been shown to stimulate erythropoietin production in patients with multiple myeloma. Aerbajinai *et al* showed that thalidomide induced  $\gamma$ -globin mRNA expression in a dose-dependent manner, but had no effect on  $\beta$ -globin expression (Aerbajinai, *et al* 2007). A trial to ascertain the tolerability and efficacy of pomalidomide in SCD (NCT: 01522547) has recently closed and initial results presented at the American Society of Hematology annual

meeting were promising with the drug being well tolerated in SCD. A phase 2 trial is in planning.

#### **1.5.2.2 Transplantation**

Bone marrow transplantation (BMT) is currently the only curative treatment for SCD. Despite massive potential benefits there are also substantial risks attached to this process. The main risks are a) infection during the chemotherapy and neutropenia of transplant, b) graft versus host disease, c) infertility, d) graft rejection and e) reactivation of viral infections. Due to the potential for long-term severe side effects from transplantation and a 3-7% risk of mortality attached to the procedure patient selection is important (Bernaudin, *et al* 2007, Hsieh, *et al* 2011, Hsieh, *et al* 2009, Panepinto, *et al* 2007) with the majority of BMT performed to date in patients under 18 years of age with evidence of severe disease (such as recurrent vaso-occlusive episodes or stroke) and a fully matched sibling donor. Currently reduced intensity conditioning regimes are being developed for adult patients who have a higher burden of end organ damage (Bolanos-Meade, *et al* 2012, Hsieh, *et al* 2011).

Various chemotherapy protocols are used to prepare the patient for transplant but all result in some degree of neutropenia and the resultant sepsis can be life threatening. Graft versus host (GvH) disease is when the new immune system from the donor recognises the patient as a foreign antigen and develops an immune response against it. The lowest risk of developing GvH is in an HLA-matched sibling donor - unfortunately only a minority of patients have a donor unaffected by SCD. There is therefore ongoing research into the use of alternative sources of stem cells such as cord blood or unrelated donors including haploidentical transplants (NCT: 00977691, NCT: 01461837).

#### **1.5.2.3 Vasodilators**

As previously described (1.2) SCD is characterised by chronic haemolysis and periodic vaso-occlusion. It is therefore logical that drugs causing vaso-dilation could be beneficial, to prevent or treat painful episodes.

### **Inhaled Nitric Oxide**

Nitric oxide has a key role in the control of vasodilation. Haemolysis of sickled RBCs leads to consumption of NO and therefore endothelial dysfunction and vasoconstriction. Thus direct supplementation of NO could in theory reverse this imbalance. In 2003 *Weiner et al* carried out a prospective double-blinded placebo controlled trial comparing inhaled NO and oxygen with placebo (21% oxygen) for treatment of vaso-occlusive crisis. There was no significant decrease in pain as measured by a visual analogue scale. Morphine use however did reduce significantly (Weiner and Brugnara 2003). There is currently one phase II study ongoing for use of inhaled NO in acute painful crisis (NCT: 00142051 - paediatric patients) and also one study looking at its role in acute chest syndrome (NCT: 01089439). Another trial examining the role of NO in ACS in adults has closed but no data is yet available. NO should not be used long-term however due to the risk of peripheral neuropathy and megaloblastic anaemia (Ogundipe, *et al* 1999).

### **Sildenafil**

Sildenafil is an oral phosphodiesterase-5 inhibitor initially marketed as a treatment for impotence. Sildenafil amplifies NO signalling via inhibiting the hydrolysis of cGMP in tissues that express phosphodiesterase-5 (e.g. lungs and corpora cavernosa) thus leading to vasodilatation. It has been used successfully in the treatment and prevention of priapism in SCD patients and has been successfully used for many years in the treatment of pulmonary hypertension not associated with SCD. A series of 12 patients with SCD and PHT were successfully treated with sildenafil with improvement in their tricuspid velocity gradient (Machado, *et al* 2005). Unfortunately a multicentre trial in PHT associated with SCD (NCT: 00492531) was terminated early due to increased adverse events in the sildenafil arm; the treated patients had 30% more painful vaso-occlusive crises compared to those receiving placebo. The reasons for this unexpected finding are unclear (Machado, *et al* 2011). Villagra *et al* have shown that sildenafil reduced platelet activation (Villagra, *et al* 2007). This suggests an interaction between haemolysis, decreased NO bioavailability, and platelet activation contributing to pulmonary hypertension in SCD. Another study in patients with priapism was terminated due to lack of funding (NCT: 0094090), however results appear to show a decrease in major priapism episodes in the arm treated with sildenafil although only 13 patients were enrolled in total (Burnett, *et al* 2014).

## **Arginine**

As previously described even in steady state patients with SCD have a degree of haemolysis. This leads to a chronic release of arginase, leading to decreased arginine levels, which in turn depletes NO leading to increased vasoconstriction. A multicentre, double blind placebo controlled phase II study (NCT: 00513617) of oral arginine supplementation was disappointing, showing no increase in arginine level or change in NO level or RBC density. A phase II study (NCT: 0002973) into its effects in ACS continues to recruit.

## **Statins**

These cholesterol reducing agents have become ubiquitous in general medicine. As well as their ability to lower cholesterol they also have other vasculo-protective abilities including the up regulation of endothelial NO. A pilot study (NCT: 00508027) compared 2 different doses of simvastatin on biomarkers of vasoreactivity, endothelial adhesion and inflammation at escalating doses and assessed its tolerability in patients with SCD. The investigators showed that NO levels were significantly increased from baseline by both doses, CRP was decreased in both dose groups as were levels of IL-6 (Hoppe, *et al* 2011). This has led to another study (NCT: 00508027) which is currently recruiting.

### **1.5.2.4 Prevention of red cell dehydration**

The sickling of RBCs is critically dependent on the intracellular concentration of deoxygenated HbS. The higher the concentration of HbS the shorter the time to polymerisation and therefore sickling - dehydrated RBCs have the highest concentration of intracellular HbS. There are three pathways which decrease RBC hydration 1) Gardos channel 2) the KCl transporter and 3) the Na<sup>+</sup> pump. By inhibiting any of these pathways there is theoretical clinical benefit.

### **ICA-17043 (Senicapoc)**

ICA-17043 acts via decreasing Gardos channel activity and calcium induced K<sup>+</sup> efflux. Due to promising in vitro effects in mice and humans and a long half-life enabling easy dosing a phase II randomised clinical trial was commenced. Unfortunately despite significant decrease in haemolysis (as evidenced by LDH, bilirubin and reticulocyte count) there was no significant change in painful events leading to termination of the phase III study (Ataga, *et al* 2011). There are no trials ongoing with ICA-17043.

## **Magnesium**

Magnesium regulates many cellular cation transporters including the KCl co-transporter linked to cell hydration. Raised levels of intracellular  $Mg^{2+}$  cause inhibition of  $K^+$  efflux thus preventing RBC dehydration. There has been one completed Phase I trial to estimate the maximum tolerated dose of magnesium pidolate in combination with hydroxyurea (Hankins, *et al* 2008). The Magnesium in Crisis (MagIC study: NCT: 01197417) is ongoing, examining the effect of magnesium on length of stay in the paediatric population.

### **1.5.3 Organ Specific Treatment**

#### **1.5.3.1 ACE and Angiotensin 2 Inhibitors**

The use of angiotensin-converting enzyme (ACE) inhibitors and their role in control microalbuminuria in conditions such as diabetes is well documented (Casas, *et al* 2005, Maione, *et al* 2011, Ravera, *et al* 2007). Similar results have been shown to reduce levels of proteinuria in small-scale trials (Falk, *et al* 1992) in SCD. A trial is recruiting examining the ability of losartan to improve renal function in patients with SCD (NCT: 01989078).

#### **1.5.3.2 Iron Chelation**

The reported prevalence has not been fully established, although Ballas *et al* report that >50% of patients have a transferrin saturation of greater than 50% in about 30% of patients with SCD (Ballas 2001). Despite the fact that in SCD iron does not appear to accumulate in the heart it does appear to lead to liver damage and consequent mortality (Darbari, *et al* 2006, Wood, *et al* 2004). Therefore treatment of iron overload is required. Three iron chelators are used in SCD. Deferoxamine (intravenous or sub-cutaneous administration), Deferiprone and Deferasirox (both oral administration). A recent review by Lucania *et al* raised concerns that the current usage of chelation treatment in SCD was not based on strong evidence of effectiveness (Lucania, *et al* 2011). Cochrane currently recommends deferoxamine to be used as first line treatment for iron overload in SCD and deferasirox to be reserved for patients with low-compliance or side-effects to deferoxamine (Meerpohl, *et al* 2010, Roberts, *et al* 2005).

#### **1.5.3.3 Ursodeoxycholic Acid**

Ursodeoxycholic acid is a bile acid. It is commonly used in the treatment of primary biliary cirrhosis, an autoimmune liver disease. Ursodeoxycholic acid appears to improve biliary flow

within the liver and in primary biliary cirrhosis has been shown to improve liver function and reduce jaundice and ascites, although it does not decrease mortality or liver transplantation (Gong, *et al* 2008). It is therefore used for symptomatic treatment in sickle hepatopathy.

## **1.6 Aims and Objectives**

Sickle Cell Disease presents a complex phenotype. Elucidation of the causes for this requires an accurately described cohort enabling further understanding of the pathophysiology of SCD and to establish predictors (genetic and otherwise) of specific complications. This in turn leads to the possibility of developing new treatment strategies (based on understanding pathophysiology) and pre-emptive diagnosis of complications (and development of screening tests) allowing early intervention.

### **1.6.1 Aim**

To increase our understanding of the pathophysiology of sickle cell disease, identify modifiers of disease severity and to define predictors of organ-specific complications in SCD.

### **1.6.2 Objectives**

- 1)** To accurately characterise a cohort of SCD patients at King's College Hospital (KCH) in relation to clinical history and biological variables for use in laboratory work and observational studies (Chapter 3).
- 2)** To further understand pathophysiology of SCD through investigation of genetic and biological markers:
  - a)** To measure relative telomere length in white blood cells in adult patients with SCD and its correlation, if any, with other markers (Chapter 4).
  - b)** To assess the role of Duffy genotype and derived phenotype on biological variables and markers of clinical severity in SCD (Chapter 5).
- 3)** To elucidate predictors of end-organ complications:
  - a)** Renal: To investigate associations between renal function and candidate genes (*APOL1*, *DARC* and *HMOX1*) (Chapter 6).
  - b)** Liver: To evaluate non-invasive techniques as methods of assessing liver dysfunction in SCD and to assess their value predictors for the development of liver dysfunction (Chapter 7).



## Chapter 2 : Description of Statistics Used in Thesis

### Simple analysis

Simple statistical tests are used to compare two different population groups to show a difference between them, or to show associations between two different variables. Such tests are unable to utilise all the data points pertaining to an individual, instead being reliant on “compound” measurements being produced (e.g. mean or representative data being chosen). These methods also do not enable examination of how parameters change within an individual (e.g. over time) or the influence of inter-individual differences to be incorporated into the analysis (e.g. the effect of gender or sickle genotype).

### Student's T test

The independent samples *t*-test was used in the thesis to compare the means between separate groups of patients (e.g. WBCs in Duffy positive and Duffy negative patients with sickle cell disease). The *t*-test is calculated as shown below:

$$t = \frac{\text{observed difference between sample means} - \text{expected difference between population means}}{\text{estimate of the standard error of the difference between 2 sample means}}$$

The expectation is that the difference will be zero (or close to zero) if there is no difference between the two populations being examined. The populations should be normally distributed and the assumption is also made that the variance between the two groups is the same (although this can be corrected for). Outliers can also skew the reliability of the *t*-test. In this case the Mann-Whitney U test (a non-parametric test) can be used as it is reliant on fewer assumptions in assessing differences between the two groups. Its strength is that it is a straightforward method of comparing two groups of data, and requires only one data point from each subject (although this does mean that the power of longitudinal data, with multiple data points per subject, is lost).

### Wilcoxon rank-sum test and Mann-Whitney U test

These tests were used in the thesis to compare separate groups of patients where there was significant variance between the groups, or when the data was not normally distributed. These tests rely on the ranking of all the data in both groups from lowest to highest. If there is no difference between the groups then the “sum” of both groups’ rankings will be the same, or

nearly the same. The weakness of these tests is that they have a lower power than parametric tests, and therefore it is more difficult to find smaller differences between two populations.

### **Chi squared test and Fisher's exact test**

The Chi-squared test and Fisher's exact test were used in the thesis to examine expected vs observed frequencies of clinical complications and other categorical data. It is assumed that the expected frequencies will not be different between the groups and deviation from this in the observed data is significant. One of the limitations is that all participants measured must be independent, meaning that an individual cannot fit in more than one category. Another consideration is that the chi-square statistic is sensitive to sample size. Most recommend that chi-square not be used if the sample size is less than 50, in which case the Fisher's exact test is more appropriate. The chi-square test does not give information about the strength of the relationship between two variables, rather that the association exists. The main strength of the chi-square test is that it is a simple statistical method of showing association and can be used with categorical (or nominal data); it also (unlike other statistical methods) makes no assumptions about the distribution of data (e.g. it is not reliant on data being normally distributed).

### **Pearson's and Spearman's correlation coefficient**

Pearson's correlation coefficient is a standardised measure of the strength of a relationship between two continuous variables. The larger the  $r$  value, the stronger the association. It relies on the data being normally distributed; in contrast Spearman's correlation coefficient does not rely on the assumptions of a parametric test. Data are normalised by transformation into ranked scores and then a Pearson's test is performed. However, having a large correlation coefficient does not imply causality, merely association. The other weakness with this method is that if the relationship is not linear, then the result is inaccurate.

## **Model based analysis**

For more complex analysis of the data sets, and the relationships within them, Dr Fulford performed linear and logistic regression.

### **Linear and logistic regression**

Linear regression was used in the thesis to examine the association between predictor variables (e.g. laboratory results) and outcome variables (e.g. telomere length). Linear regression results from creating models to summarise the relationship between one or more predictor variables and an outcome variable. The assumptions are that the outcome variable should be linearly related to any predictors and, when multiple predictors are examined, that their effects should be additive. It is also assumed that there is no multi-collinearity, i.e. that the predictor variables are not significantly associated with each other (e.g. liver function tests will correlate with each other, as well as the outcome variable under examination). However, simple regression does allow the strength of the association between variables to be assessed via the size of the coefficient related to that variable. As with the *t*-test, outliers can significantly affect the estimates of the regression co-efficients, causing bias. It is also important that the residuals resulting from the regression should be normally distributed for the model to be a good fit. Logistic regression is used when the outcome is a categorical variable (e.g. presence or absence of albuminuria).

The strengths of this method enable the using of all data points relating to an individual, rather than a mean or representative value. This gives appropriate weight to each individual e.g. a two-point mean is less reliable (and therefore of less potential significance) than a 30-point mean value. The model should also enable the examination of parameters that can change within each individual, e.g. the effect of time on albuminuria, while compensating for other variables such as gender or sickle genotype. These inter individual differences (gender, genotype etc) can also be examined for their effect on the parameter under investigation.

The main weakness of this method is that differentiating between inter and intra individual variances can sometimes be difficult, leading to problems obtaining the  $R^2$  value which is the end result of a Pearson's correlation. A more complex model can be used looking at random effects between individuals, however in a clinical situation this is not relevant as "whole group" effects are the desired output.

## Chapter 3 The King's Sickle Cohort

### 3.1 Introduction

Prior to performing genetic association studies, patient phenotypes need to be accurately defined which, includes details of demographic data, sickle and alpha-globin genotype, clinical complications and laboratory parameters. The King's sickle cohort described here is the patient pool from which the sub-cohorts for the rest of the studies in this thesis are derived, and comprises all patients who attended the specialist sickle cell clinic from January 2000 to December 2012. However any cohort description carries the warning that it is only a "snapshot" of the patient group over a particular time period. Although our patients attend KCH for the majority of their care there may be occasions when they are seen in other hospitals. We have ensured that data collection is as accurate as possible and complications have been carefully defined prior to the study commencing.

The diversity of presentations of sickle cell disease (SCD) adds to the complex nature of the cohort description. Complications as measured by end-organ damage, such as end stage renal failure as a measurement of sickle cell nephropathy are rare and therefore require a large sample size for association studies to reach significance. An alternative is to use proxy or intermediate measures such as proteinuria or glomerular filtration rate to capture those patients at risk of developing the complication. As discussed previously there have been many studies focusing on specific complications and genetic associations (Chaar, *et al* 2006b, Hoppe, *et al* 2004, Kutlar, *et al* 2001, Nolan, *et al* 2005a, Sebastiani, *et al* 2005, Ulug, *et al* 2009, Vasavda, *et al* 2007). However large cohort series looking at prevalence and incidence of multiple complications are few and limited to African-American patients (Platt, *et al* 1994, Powars, *et al* 2005). The majority of studies from the United States stem from the Cooperative Sickle Cell Disease Study Group (ten sickle cell centres which were funded by the National Institute of Health to promote research and clinical care for patients with SCD). This project was initiated in 1977 to determine the natural history SCD from birth to death with the purpose of identifying factors contributing to the morbidity and mortality of the disease. Specific objectives included: 1) studying the effect of SCD on growth and development from birth through adolescence; 2) studying the conditions or events that may be related to the onset of painful crises; 3) obtaining data on the nature, duration, and outcome of major complications of SCD; 4) determining the nature, prevalence, and age- related incidence of organ damage due to SCD, and 5) study the

role of SCD and its interaction with selected health events. Participant entry ended in 1988. Both mild and hospital-based sickle cell patients were recruited. Over 4500 patients were recruited during this time period. Participants underwent a baseline exam for assessment of demographics, prior medical history, lab assessments, and clinical data. Post baseline data included routine follow-up examinations, measures of organ damage, and collection of acute and chronic complications.

One of the weaknesses with the American studies is the lack of access to universal health care that is free at the point of demand. The reliance on a combination of public and private insurance schemes means that although children have open access to healthcare once patients transition to adult services care is less comprehensive and mortality in the 18 – 30 age group is higher than expected. Quinn *et al* demonstrated that the majority of deaths occur after 18 years of age and after transfer to an adult provider (85%) (Quinn, *et al* 2004). The American cohorts do not therefore necessarily represent the experience of the patient with SCD in a well resourced setting e.g. the UK or France.

### **3.1.1 Previous Cohort Studies**

Acute pain has been a major focus of cohort studies in SCD. Platt *et al*'s 1991 study (in a cohort of 3578 patients with mixed genotypes) reported that 5.2% of participants with HbSS had 3 – 10 episodes of pain per year and that this group accounted for about one third of all acute pain episodes in that cohort. Further, almost 40% had no episodes of pain significant enough to warrant a clinic visit (Platt, *et al* 1991) throughout the 10 year study period. This phenotypic variability extends to other complications of SCD.

A subsequent mortality study of SCD by the same group showed that only 18% of patients had chronic complications at the time of death (Platt, *et al* 1994). Two large prospective cohort studies by Powars *et al* represent some of the most significant work in understanding the morbidity and mortality burden incurred by sickle cell disease (Powars, *et al* 2005, Powars, *et al* 2002). 1056 patients with HbSS were recruited between 1959 and 2003 and studied longitudinally (Powars, *et al* 2005). Acute pain represented the most common acute complication, with 70% of patients enrolled being hospitalised with at least one acute painful episode by the end of the study period. Other common causes of hospitalisation were acute

chest syndrome (48%), hypersplenism (20%) bone infarction (15%) and aplastic crisis (14%). Chronic complications were less prevalent nonetheless significant morbidity was described throughout the cohort. Common chronic complications were gall bladder disease (28.1% of patients), avascular necrosis (21.2% of patients), sickle cell chronic lung disease (15.6%), leg ulcers (14.4%), priapism (13.5%), renal failure (11.6%), cerebrovascular accident (11.0%) and retinopathy (8.7%). The incidence of these complications rose dramatically in the 232 patients for whom they had recorded deaths. The presence of one chronic complication resulted in statistically significantly increased risk of developing further complications (Powars, *et al* 2005).

Cox regression curves showed significantly improved childhood survival in patients born after 1975 coinciding with improved awareness of infections and vaccines, as well as latterly the promotion of prophylactic penicillin. Major causes of death were chronic lung disease with pulmonary hypertension and cor pulmonale, chronic renal failure and cerebral vascular accident (Powars, *et al* 2005).

Powars *et al* concurrently collected data from Hb SC patients (n= 284) (Powars, *et al* 2002). They reported lower incidence of all complications bar retinopathy than in HbSS patients (Powars, *et al* 2002). Retinopathy was the most common complication being present in 22.9% of patients, followed by osteonecrosis (14.8%), gall bladder disease (7.8%) and CVA (4.6%) (Powars, *et al* 2002). The Powars' data suggests that 50% of children born with HbSS in the 21<sup>st</sup> century will live into their fifth decade carrying significant morbidity burdens (Powars, *et al* 2005).

Thomas *et al* published the Jamaican experience in 1982 focusing on causes of death in 276 patients (241 Hb SS, 23 Hb SC, 7 Hb S $\beta$ <sup>+</sup>, 4 Hb S $\beta$ <sup>o</sup> and 1 Hb SOArab) with an age range of 1 month to 76 years over a 30 year period from 1952 to 1982. The majority of deaths occurred in the first 5 years of life, with roughly one-quarter occurring in the 30 plus age group. However, this cohort included patients who were not routinely started on prophylactic penicillin, now standard of care, and known to significantly reduce mortality in the paediatric group. The most common cause of death in all groups was acute chest syndrome.

## **3.2 Objectives**

**To accurately characterise a cohort of SCD patients attending King's College Hospital (KCH) between January 2000 to December 2012, in relation to clinical history and laboratory variables for use in laboratory work and observational studies**

## **3.3 Methods**

### **3.3.1 Data Collection**

Data collection was from a variety of sources and was collected on all sickle cell patients registered at KCH. Data was stored on the sickle cell database (developed by Professor Thein and Teleologic systems, Norwich, UK) and Caldecott principles were strictly adhered to. The sickle cell database is a clinical and research register of all patients who have attended King's College Hospital from 2001 to the present day.

Clinical data was collected from June 2011 to December 2012 (ongoing) and was gathered retrospectively and prospectively. Retrospective data was collected using past medical history review forms (see appendix) during the routine steady state clinic visits. Hard evidence of complications including results of radiological investigations was available from 1999 to the present on the electronic patient record (EPR). Evidence of complications was validated by appropriate radiological investigations or clinical examinations e.g. ophthalmological review. Prospective data was gathered using steady-state review forms (see appendix) and entered directly into the database (when staff had received training) to record the development of new complications and the number of pain episodes since the last clinic visit. Complication data collected included avascular necrosis (AVN), osteomyelitis, acute chest syndrome (ACS), tricuspid regurgitant jet velocity >2.5 m/s, sickle cell lung disease (SCLD), stroke (ischaemic and haemorrhagic), retinopathy (symptomatic and asymptomatic), gallstones (symptomatic and asymptomatic), priapism, leg ulcers and renal impairment. Renal impairment was defined by presence of microalbuminuria (albumin:creatinine ratio [ACR] greater than or equal to 4.5), macroalbuminuria (ACR greater than or equal to 30 – equivalent to 1+ protein on urine dipstick) or an estimated glomerular filtration rate (eGFR) of less than 60 mL/min/1.73 m<sup>2</sup> (stages 3-5 of chronic kidney disease).

Sickle cell genotypes were determined by high performance liquid chromatography (Hb SS and Hb SC) and sequencing of the beta-globin locus to confirm the genotype of the other  $\beta$  globin allele in compound heterozygotes (Hb S $\beta$ +, Hb S $\beta$ <sup>0</sup> and Hb SHPFH). Common alpha deletion mutations (3.7, 4.2, SEA, 20.5, MED, FIL) were identified by multiplex gap-PCR. These tests were performed by the King's College Hospital Red Cell Centre laboratory. All results were confirmed by two separate clinicians prior to entry on the sickle cell database.

Laboratory data sets were generated automatically via a direct download from the electronic patient record to the sickle cell database, for all patients, for each steady state visit from January 2005 to December 2012. Electronic transfer minimises transcription errors and also ensures laboratory data were recorded concurrently with the appropriate clinical visit. Laboratory variables collected are shown in Table 2 including units and normal ranges for our laboratory.

Patient samples following transfusion within 6 weeks and samples taken while on hydroxycarbamide was also recorded. Transfusion data was collected from January 1990 to December 2012 by Dr N Igbineweka and myself from the transfusion databases at KCH (PathNet, Winpath and EPR) and recorded on the sickle cell database.



Table 2: Laboratory variables recorded on sickle cell database, their units and normal ranges for the King's College Hospital laboratory.

Haematology tests were performed on the Advia® 2120i haematology analyser, biochemistry on the Advia Centaur® XP immunoassay system (both Siemens Surrey, UK), coagulation tests on the Stago star evolution (Reading, UK)

Laboratory variable	Units	Normal range
Haemoglobin (Hb)	g/L	130 – 165
Mean cell volume (MCV)	fL	77.0 – 95.0
Mean cell haemoglobin (MCH)	pg	25 – 34
White blood cell count (WBC)	$\times 10^9/\text{L}$	4.00 – 11.00
Neutrophil count	$\times 10^9/\text{L}$	2.2 – 6.3
Reticulocyte count		
Platelets	$\times 10^9/\text{L}$	150 – 450
Serum ferritin	ng/L	20 – 200
Iron saturation	%	
Total iron binding capacity (TIBC)	umol/L	50 – 72
Soluble transferrin receptor (sTR)	nmol/L	8.7 – 28.1
Erythropoietin (EPO)	IU/L	5 – 25
Sodium (Na)	mmol/L	135 – 145
Potassium (K)	mmol/L	3.5 – 5.0
Urea	mmol/L	3.3 – 6.7
Creatinine	mmol/L	45 – 120
Albumin	g/L	35 – 50
Alanine transaminase (ALT)	IU/L	5 – 55
Aspartate transaminase (AST)	IU/L	10 – 50
Alkaline phosphatase (ALP)	IU/L	20 – 130
Gamma-glutamyl transpeptidase (GGT)	IU/L	1 – 55
Corrected calcium	mmol/L	2.15 – 2.6
Cystatin C	ng/L	less than 1.4
Albumin: creatinine ratio	N/A	< 4.5
Protein : creatinine ratio	N/A	< 15
CRP	mg/L	less than 5
Haemoglobin S%	%	
Haemoglobin F%	%	

N/A = not applicable

### 3.3.2 Definitions of Sickle-related Complications

The definition of the clinical complications had to be accurate to prevent “no data” points becoming falsely recorded as negative. Table 3 summarises the clinical and radiological proofs required for a positive (or negative) complication to be recorded.

The diagnosis of end-organ damage is influenced by changes in clinical practice. Whilst some complications (e.g. avascular necrosis) are only looked for when symptoms occur, others are screened for routinely in specific demographic groups (e.g. echocardiogram screening for pulmonary hypertension in all patients over 50) or in conjunction with specific studies ongoing

during the study period. This may well increase the apparent incidence of some end-organ complications in asymptomatic individuals, and lead to the under-diagnosis of others that are not routinely screened for. Whether or not this affects the outcome of individual patients is a matter for further study.

Table 3: Summary of clinical and investigative evidence required for a positive sickle-related complication to be recorded on the Sickle Cell Database

<b>Complication</b>	<b>Clinical Evidence</b>	<b>Radiological/laboratory/ Echocardiogram Evidence</b>
<b>Avascular necrosis</b>	N/A	Positive findings on MRI or X ray
<b>Osteomyelitis</b>	Patient report	Positive findings on MRI
<b>Acute chest syndrome</b>	Patient report	Positive CT or X ray findings
<b>Raised TRJet &gt;2.5 m/s</b>	N/A	TRJet > 2.5 m/s on trans-thoracic echocardiogram or raised right heart pressures on cardiac catheter
<b>Sickle cell lung disease</b>	N/A	Positive report by radiologist on CT chest
<b>Stroke</b>	Patient report	Positive MRI, CT or MRA findings
<b>Retinopathy</b>	N/A	Positive findings on retinal examination by ophthalmology
<b>Symptomatic retinopathy</b>	Patient reporting visual disturbance	Vitreous haemorrhage or retinal detachment on examination by ophthalmology
<b>Gallstones</b>	N/A	Ultrasound evidence of gallstones
<b>Symptomatic gallstones</b>	Patient reported cholecystitis or cholecystectomy	Ultrasound evidence of cholecystectomy
<b>Priapism</b>	Patient report	N/A
<b>Leg ulcers</b>	Patient report	N/A
<b>Microalbuminuria</b>	N/A	ACR more than or equal to 4.5
<b>Macroalbuminuria</b>	N/A	ACR more than or equal to 30
<b>MDRD eGFR ≤60</b>	N/A	Less than 60 mL/min/1.73 m <sup>2</sup>

CT = computerised tomography MRI = magnetic resonance imaging MRA = magnetic resonance angiography  
TRJet = Tricuspid regurgitant jet velocity MDRD eGFR = Modification of diet in renal disease estimated glomerular filtration rate N/A = not applicable

### 3.3.3 Deaths

Causes and ages of death were recorded where available. Some patients left our service and have been lost to follow-up, thus survival data is unavailable. Some patients died in the community and letters were written to general practitioners to ascertain causes of death, although response rates were poor.

## **3.4 Results and Data Analysis**

### **3.4.1 Demographics of King's Sickle Cell Cohort**

796 patients were recorded on the sickle cell database from 01/01/2000 to 31/12/2012 (13 years). These patients were followed up for a total of 5341 patient-years, with a mean follow-up per patient of 6.7 years (range 1 - 13).

As shown in Figure 3, a minority of our patient group (99/796, 12%) have attended at King's for the entire 13 year study period. Some patients, once they are referred to the adult service, are regular attendees, others may not for years at a time.

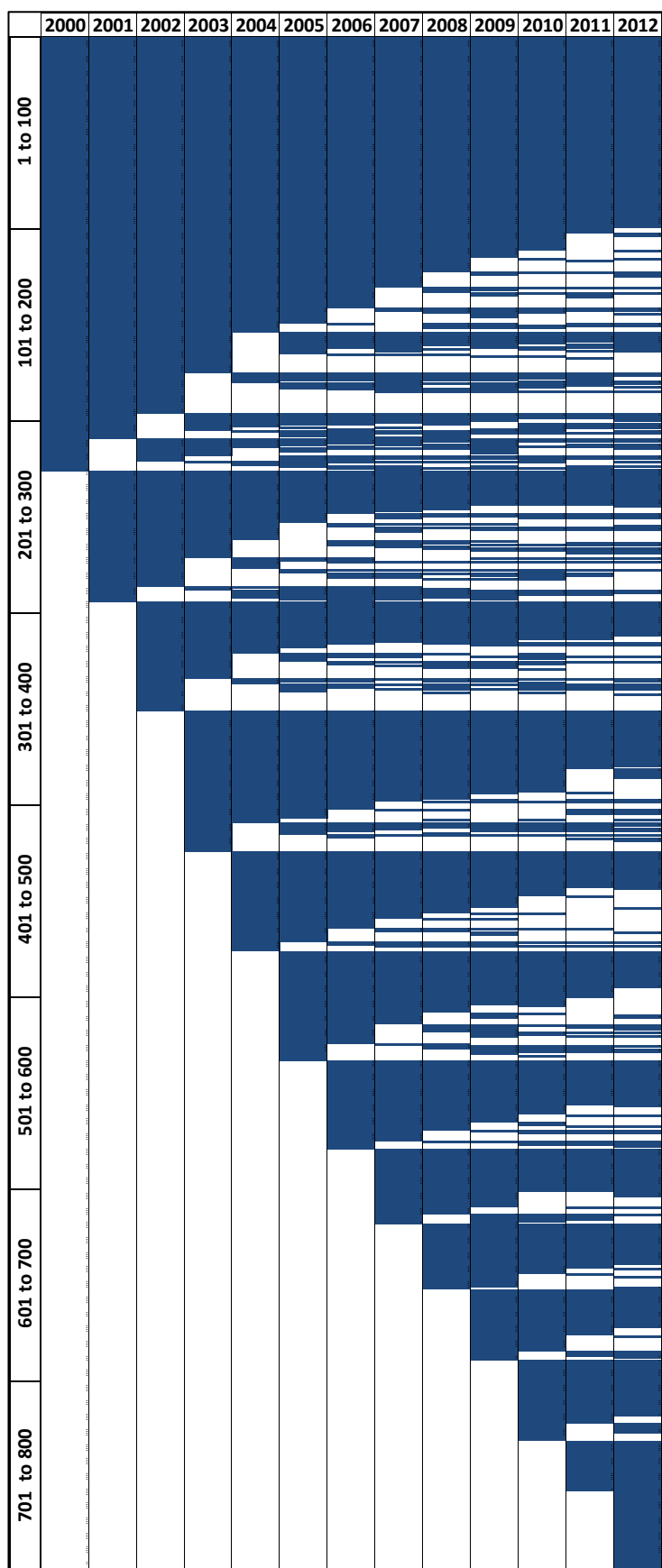


Figure 3: Figure showing the transitory nature of our sickle cell cohort over a thirteen year period. Each horizontal line represents a single patient with the vertical coloured bars representing a contact with the department on a yearly basis, representing 5341 patient years in total

Despite the transitory nature of our cohort, patient numbers have increased over the study period from 225 in 2000 to 515 in 2012. In the last 3 years the population has remained between 450 and 500 (see Figure 4). However the proportions of the individual genotypes remain similar despite the increase in patient numbers mean Hb SS 62% (range 57 – 65%), mean Hb SC 32% (range 27 – 36%), mean Hb S $\beta^+$  5% (range 4-5%), mean Hb S $\beta^0$  1% (consistent) and mean Hb SHPFH <1% (range 0-1%).

Figure 5 illustrates the decreasing proportion of the more severe sickle genotypes (Hb SS and Hb S $\beta^0$ ) with increasing age. 451/796 (57%) of our cohort were female with 345 (43%) male. These proportions were similar across all the sickle genotypes. Mean age over the study period increased from 32 to 36 years old with a range of 16 to 83 years over the study period.

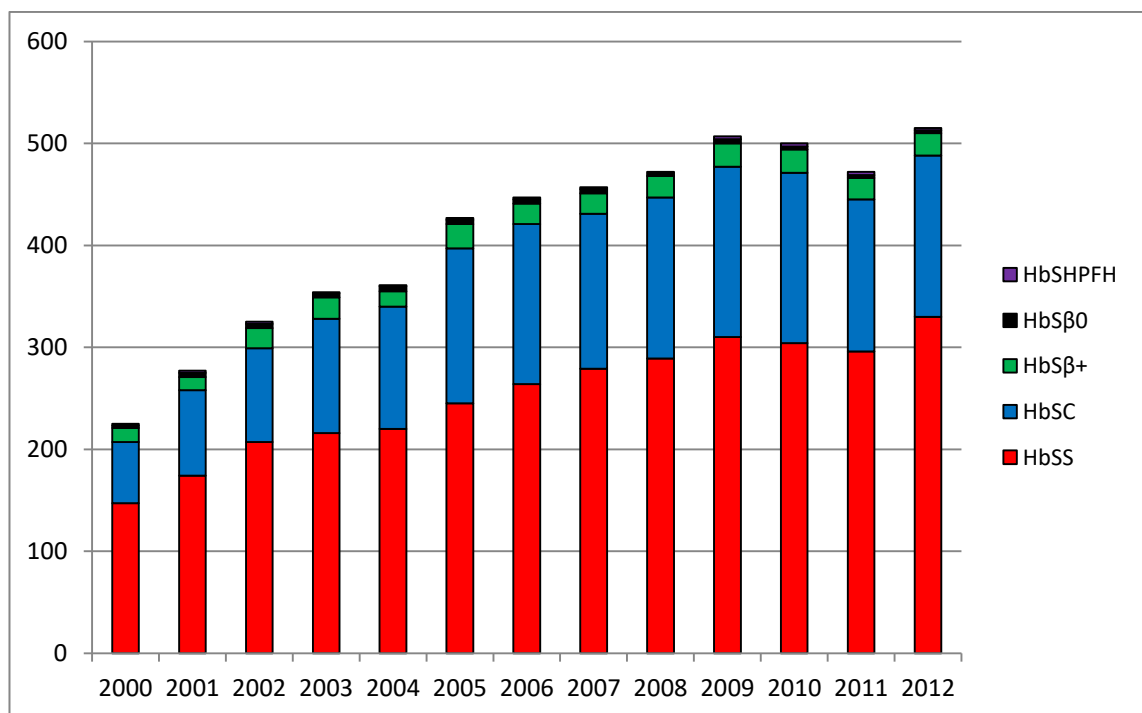


Figure 4: Total patient number and number of patients in each sickle genotype from 2000 to 2012. Patient numbers increased over the study period from 225 in 2000 to 515 in 2012. The proportions of the individual genotypes have remained similar

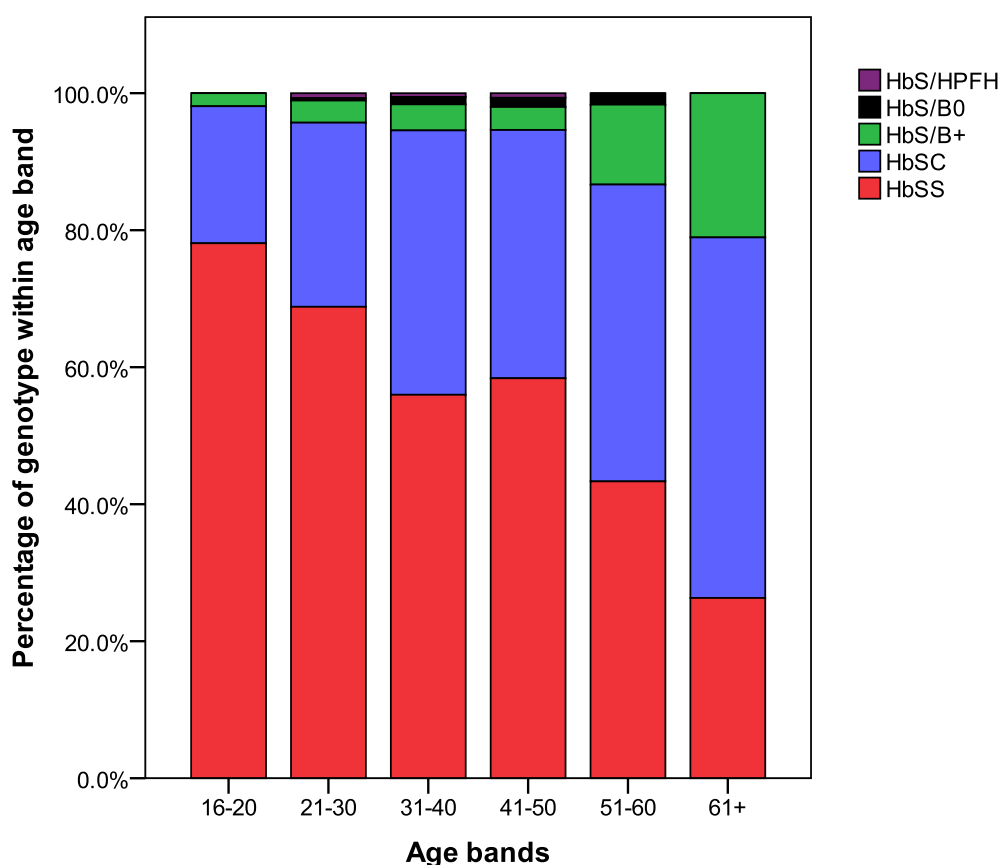


Figure 5: Percentage of sickle genotypes per age band. Age was recorded at last clinic attendance. This figure illustrates the decreasing proportion of the more severe sickle genotypes (Hb SS and Hb S $\beta^0$ ) with increasing age

### 3.4.2 Treatments: Transfusion and Hydroxycarbamide

The two commonly used treatments for ameliorating the acute and chronic complications of SCD are transfusion (acute, sporadic or programmed) and hydroxycarbamide (HC).

In the study period 59 patients (all Hb SS or Hb S $\beta^0$ ) were commenced on HC. The most common reason for treatment was acute chest syndrome, followed by frequent acute pain. 49 patients continued to receive HC at the end of the study period. Mean duration of treatment was 4 years 8 months, with a range from 4 months to 18 years, 2 patients having been commenced on HC prior to the start of the study period. 8 patients had stopped HC treatment by the end of the study period. 2 had become pregnant and not restarted treatment *post-partum*, 4 had switched to transfusion programmes and 2 stopped due to poor compliance and it was not reintroduced. 2 patients died whilst on HC treatment, one from complications resulting from pulmonary hypertension and the second from chronic renal failure secondary to sickle cell disease.

Transfusion was common in our cohort with 46% of patients having received at least 1 unit of blood during the study period. The mean units transfused during the 13 year period were 21, range of 1 – 1069 units.

A subsection of these transfusion data have been published (Drasar, *et al* 2011); see appendix

### **3.4.3 Sickle-related Complications**

Sickle-related complications were recorded on the sickle cell database as defined in Table 3. The prevalence of conditions was taken from the existence or past occurrence of a complication at the end of the study period. Due to only small numbers of patients having Hb S $\beta^+$  and Hb SHPFH, further analysis was restricted to Hb SS and Hb S $\beta^0$  (considered as sickle cell anaemia, SCA) and Hb SC, and analysis of priapism data was restricted to male patients only. Differences in prevalence of sickle-related complications between the genotypes were examined using the Chi-squared test and binary logistic regression (corrected for age, alpha-genotype and gender). Gallstones were the most common complication across the entire cohort, however only 60% of these were symptomatic. The prevalence of all complications increased with increasing age. The majority of complications were more common in SCA patients compared to Hb SC, significantly so for acute chest syndrome, pulmonary hypertension, sickle cell lung disease, stroke, gallstones, raised ACR and leg ulcers. This significance remained despite correction for age. An increased prevalence of MDRD eGFR less than 60 mL/min/1.73 m<sup>2</sup> was found in patients with SCA only once a correction had been made for by age. Exceptions include avascular necrosis, present in similar proportions in SCA and Hb SC patients. Another exception to end-organ damage being more common in SCA is sickle retinopathy which was found in 50% of patients with Hb SC who had been formally assessed by an ophthalmologist, in contrast to only 18% of patients with SCA. The results are summarised in Table 4.

Table 4: Prevalence of specific sickle-related complications for whole cohort, Sickle Cell Anaemia (SCA) and Hb SC at the end of the study period. Analysis refers to comparison between differing prevalence of complications between SCA and Hb SC. Not all patients within the individual patient groups had complete data available for all complications.

<b>Complication</b>	<b>Whole cohort (all sickle genotypes) cases/valid records (% cases)</b>	<b>SCA (Hb SS and Hb Sp<sup>0</sup>) cases/valid records (% cases)</b>	<b>Hb SC cases/valid records (% cases)</b>	<b>Simple analysis Chi-square p value</b>	<b>Multiple regression* Wald p value</b>
<b>Avascular necrosis</b>	85/367 (23)	57/239 (24)	25/110 (23)	N/S	N/S
<b>Osteomyelitis</b>	25/401 (6)	19/264 (7)	6/117 (5)	N/S	N/S
<b>Acute chest syndrome</b>	86/425 (20)	76/278 (27)	9/124 (7)	20.74 <b>p&lt;0.0001</b>	10.1 <b>p=0.001</b>
<b>TRJet ≥2.5</b>	53/318 (17)	44/210 (21)	8/92 (9)	6.74 <b>p=0.009</b>	5.88 <b>p=0.001</b>
<b>Sickle cell lung disease</b>	38/274 (14)	35/187 (19)	1/75 (1)	13.65 <b>p&lt;0.0001</b>	12.49 <b>p&lt;0.0001</b>
<b>Stroke</b>	61/456 (13)	51/396 (17)	10/137 (7)	7.63 <b>p=0.006</b>	2.59 <b>p=0.02</b>
<b>Retinopathy</b>	124/422 (29)	45/248 (18)	77/153 (50)	46.30 <b>p&lt;0.0001</b>	35.2 <b>p&lt;0.0001</b>
<b>Symptomatic retinopathy</b>	66/124 (53)	20/45 (44)	46/77 (60)	N/S	N/S
<b>Gallstones</b>	173/359 (48)	146/257 (57)	26/87 (30)	18.85 <b>p&lt;0.0001</b>	17.17 <b>p&lt;0.0001</b>
<b>Symptomatic gallstones</b>	103/173 (60)	92/146 (63)	11/26 (42)	N/S	N/S
<b>Priapism</b>	11/101 (11)	9/73 (12)	2/23 (9)	N/S	N/S
<b>Leg ulcers</b>	19/225 (8)	18/162 (11)	1/56 (2)	8.67 <b>p=0.003</b>	5.67 <b>p=0.02</b>
<b>ACR ≥4.5</b>	163/482 (34)	137/313 (44)	26/143 (15)	39.5 <b>p&lt;0.0001</b>	37.1 <b>p&lt;0.0001</b>
<b>MDRD GFR ≤60</b>	31/501(6)	25/334 (8)	6/167 (4)	N/S	6.9 <b>p&lt;0.009</b>

N/S = non-significant. \*Regression corrected for age, gender, and alpha-genotypes

The total number of sickle-related complications per patient was calculated using the date last seen as the end point of data collection. Complications included in this analysis were the same as those in Powars *et al's* 2005 paper i.e. avascular necrosis, gallstones, sickle cell lung disease, leg ulcers, renal failure (GFR ≤60), CVA (any type), symptomatic retinopathy, and priapism. 55% of patients had no evidence of sickle-related end organ complications, 29% had 1 organ involved, 12% had damage to 2 organs, 3% had damage to 3 organs, 1% had damage to 4 organs, and less than 1% had damage to 5 organs or more. As shown in Figure 6, patients



with SCA had significantly more complications than patients with Hb SC ( $p < 0.0001$ ). This persisted when corrected for age ( $\beta = 0.58$   $p < 0.0001$ ).

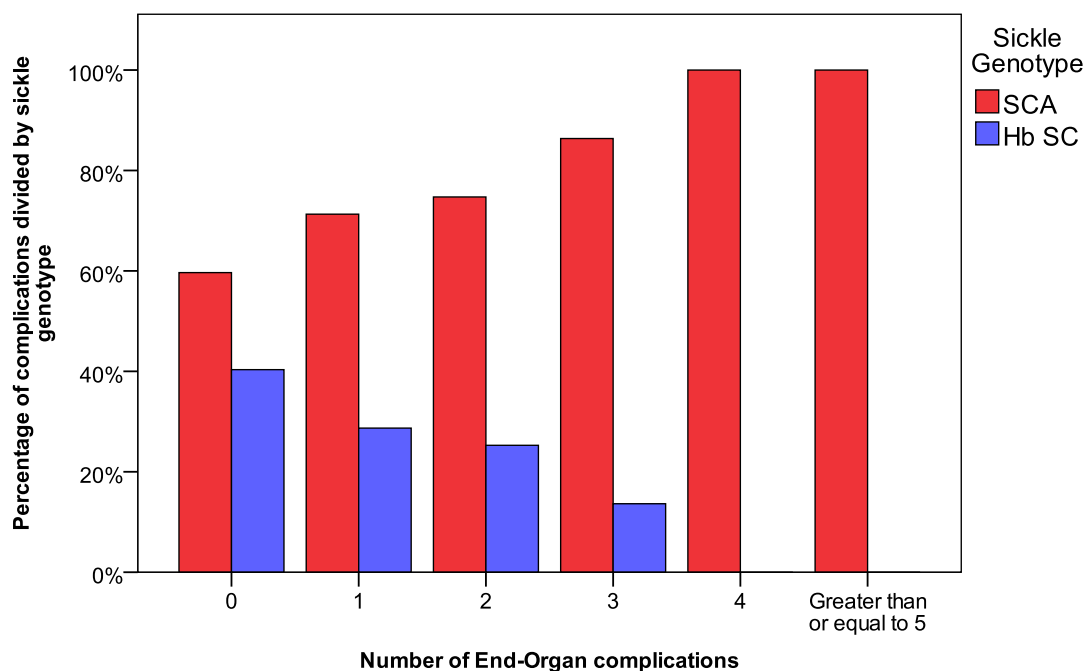


Figure 6: Percentage of end-organ complications within each sub-group of sickle genotype. Only patients with SCA were found to have 4 or more complications

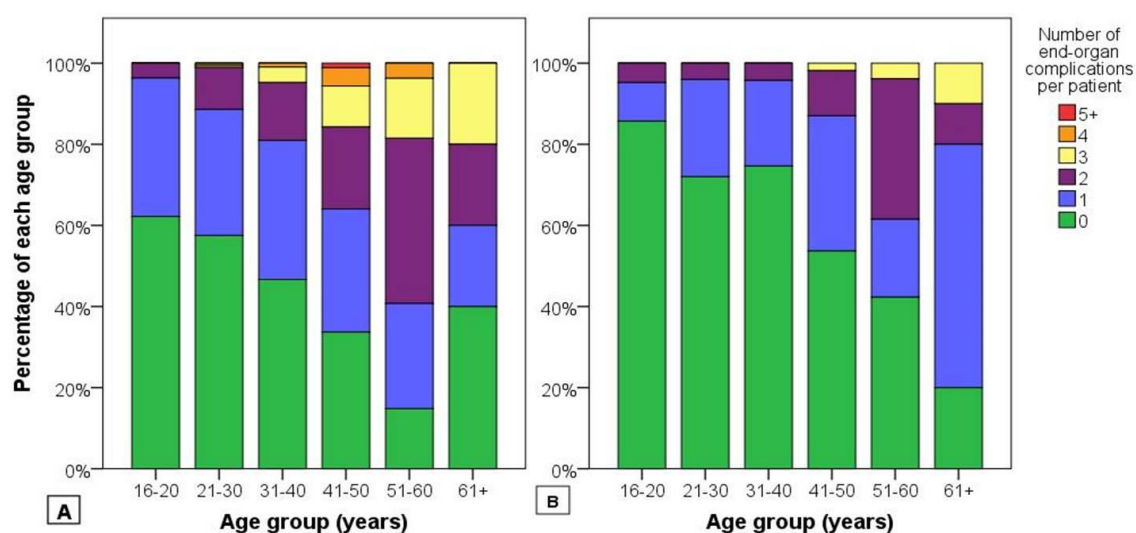


Figure 7: The number of end-organ complications within each age band. The Y axis refers to the percentage of each age band with complications. The number of complications increases with age for both SCA and Hb SC patients. A = Sickle Cell Anaemia B = Hb SC.

There appears to be a profound effect of age on the number of sickle-related complications developed. As shown in Figure 7, the number of patients without end-organ complications reduces for each age band for both SCA and Hb SC patients. 66% of SCA patients (59/89) have developed at least 1 complication by their 5<sup>th</sup> decade, in contrast to 46% of Hb SC patients (25/54). However in the SCA group the number of patients with the most complications ( $\geq 5$ ) appears to decrease as age increases. I postulate that this is due to these “severe” patients dying at an early age, whereas the older patients have inherently milder disease and have therefore survived longer. Because the Hb SC patients are a more homogeneous group, this “drop out” is less pronounced.

As our cohort ages, this may have an effect on the incidence of complications. The incidence of all sickle-related complications is summarised in Table 5. Unsurprisingly, SCA has a higher incidence of the majority of end-organ complications, again with the exception of retinopathy which is higher in Hb SC. The increased usage of interventions such as HC and transfusion may alter this data in the future and continuing to collect this data will be important with regard to future resource allocation in SCD patients.

Table 5: The incidence of sickle-related complications for a) SCA and Hb SC combined, b) patients with SCA and c) patients with Hb SC.

This is expressed as either incidence during the 13 year study period or per 100 patient years. The incidence of priapism was calculated using only male patients.

Complication	SCA and Hb SC combined		SCA (Hb SS and Hb S $\beta^0$ )		Hb SC	
	Incidence during study period	Incidence per 100 patient years	Incidence during study period	Incidence per 100 patient years	Incidence during study period	Incidence per 100 patient years
AVN	57	1.06	39	1.17	18	1.04
Osteomyelitis	12	0.22	9	0.27	3	0.17
ACS	68	1.27	59	1.77	9	0.52
TRJet $\geq 2.5$	52	0.97	44	1.32	8	0.46
SCLD	36	0.67	35	1.05	1	0.06
Stroke	41	0.76	32	0.96	9	0.52
Retinopathy	118	2.21	45	1.35	73	4.22
Gallstones	99	1.85	80	2.40	19	1.09
Priapism	9	0.40	8	0.55	1	0.16
Leg ulcers	13	0.24	13	0.39	0	0

AVN = avascular necrosis ACS = acute chest syndrome TRJet = Tricuspid regurgitant jet velocity  
SCLD = sickle cell lung disease

#### **3.4.4 Associations between complications**

As described on page 22, haemolysis and vaso-occlusion are thought to be at opposite ends of the pathological spectrum and patients are often described as having a “vaso-occlusive” or “haemolytic” phenotype depending on their presentation. We therefore looked for associations between the end-organ complications in our cohort. The Chi-squared test was used to compare pairs of complications, or Fisher’s exact test when numbers in individual groups were less than 5.

The SCA group showed a number of significant associations between specific complications. These are summarised in Table 6.

The only significant associations seen in the Hb SC group were between AVN and retinopathy (Chi-squared value 7.16  $p = 0.007$ ) and between retinopathy and gallstones (Chi-squared value 6.14  $p = 0.01$ ).

Complication	AVN	ACS	PHT	SCLD	Stroke	Retinopathy	Gallstones	ACR ≥4.5	MDRD ≤60	Priapism	Leg ulcer
AVN		N/S	N/S	5.28 p = 0.002	N/S	11.81 p = 0.001	N/S	N/S	N/S	N/S	N/S
ACS	N/S		N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
PHT	N/S	N/S		33.15 p < 0.0001	N/S	5.1 p = 0.05	N/S	7.94 p = 0.005	34.29 p < 0.0001	N/S	N/S
SCLD	5.28 p = 0.02	N/S	33.2 p < 0.0001		N/S	6.65 p = 0.01	N/S	3.65 p = 0.05	11.84 p = 0.001	N/S	N/S
Stroke	N/S	N/S	N/S	N/S		N/S	N/S	N/S	N/S	N/S	N/S
Retinopathy	11.81 p = 0.001	N/S	5.1 p = 0.05	6.65 p = 0.01	N/S		6.46 p = 0.01	N/S	N/S	N/S	N/S
Gallstones	N/S	N/S	N/S	N/S	N/S	6.46 p = 0.01		N/S	N/S	N/S	N/S
ACR ≥4.5	N/S	N/S	7.94 p = 0.005	3.65 p = 0.05	N/S	N/S	N/S		10.44 p = 0.001	N/S	5.08 p = 0.02
MDRD ≤60	N/S	N/S	34.29 p < 0.0001	11.84 p = 0.001	N/S	N/S	N/S	10.44 p = 0.001		N/S	N/S
Priapism	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S		N/S
Leg ulcers	N/S	N/S	N/S	N/S	N/S	N/S	N/S	5.08 p = 0.02	N/S	N/S	

Table 6: Sickle-related complications and associations in patients with sickle cell anaemia (SCA) only. Where significant values given increased numbers of patients with both complications were found than predicted and Chi-squared values are given.  
 AVN = avascular necrosis, ACS = acute chest syndrome, PHT = pulmonary hypertension, SCLD = sickle cell lung disease, ACR = albumin:creatinine ratio, MDRD = modification of diet in renal disease estimated glomerular filtration rate, N/S = not significant

### 3.4.5 Causes of death

34 patients died during the study period. Of these 26 had SCA, 6 Hb SC and 2 Hb S $\beta$ +. Cause of death was extracted from hospital records and from the records of the patients' general practitioners. Where available, cause of death was recorded as per the death certificates. We were able to establish a cause of death for twenty six patients. These are recorded in Table 7. The mean age of death was 41.3 (median 42, range 20 – 79) years. Excluding the patients who were known to have died from non-sickle related causes (e.g. trauma) the mean age rises to 42.8 years. The mean age for the SCA sub-group was 40.6 years (40.8 for females, 40.5 for males) and 50.3 years for the Hb SC sub-group (64.2 for females, 46.3 for men). These mean ages of death are comparable to those found by Platt in 1994 (Platt, *et al* 1994), however our numbers are considerably smaller than the number of deaths studied in that cohort.

For those with known causes of death 8 (31%) patients did not have SCD mentioned anywhere on their death certificate, even as a significant condition not contributing to death. 13/26 (50%) patients had SCD/anaemia listed as a direct cause of death (i.e. as part 1 of the death certificate). 5/26 (19%) patients had SCD/anaemia listed as a significant condition effecting the patient but not directly contributing to death (i.e. as part 2 of the death certificate).

Of the 34 patients known to have died, 18% (6) were not known to have any evidence of end-organ complications prior to their death. The majority (82%, 28) had at least one organ affected by SCD. Of the 6 patients without end-organ complications prior to death; 1 died from acute pulmonary embolus, 1 had encephalitis and 1 died from complications relating to auto-immune hepatitis. The causes of death were not known in the 3 remaining patients.

Table 7: Causes of death in our cohort within the study period.

The patients in **bold** format are those when sickle cell disease was listed as a direct contributory cause of death (i.e. part 1 on the death certificate). Part 2 of the death certificate lists significant conditions which do not directly contribute to death.

Gender	Sickle genotype	Age at death	Cause of death
M	Hb S $\beta^+$	50	1a) Adenocarcinoma of head of pancreas
F	Hb S $\beta^+$	68	1a) Carcinoma of breast
M	Hb SC	29	UNK
<b>F</b>	<b>Hb SC</b>	<b>30</b>	<b>1a) Multiorgan failure, 1b) HIV and sickle cell disease</b>
M	Hb SC	43	UNK
F	Hb SC	48	Encephalitis
M	Hb SC	67	UNK
F	Hb SC	82	1a) Pulmonary oedema, 1b) Severe left ventricular dysfunction, 1c) Aortic stenosis and hypertension
M	Hb SS	20	1a) Haemopericardium, 1b) Stab wound to heart
<b>M</b>	<b>Hb SS</b>	<b>22</b>	<b>1a) End-stage liver disease, 1b) Sickle cell disease</b>
M	Hb SS	23	1a) Bilateral pulmonary embolism, 1b) Deep vein thrombosis, 2) Sickle cell disease
M	Hb SS	24	1a) Cerebral haemorrhage
F	Hb SS	24	1a) Intracerebral haematoma
M	Hb SS	25	1a) Cerebral hypoxia, 1b) Epilepsy, 2) Acute subdural haematoma, hypothermia, disseminated intravascular coagulopathy, sickle cell disease
F	Hb SS	29	1a) Multi-organ failure, 1b) Sepsis, 1c) Autoimmune hepatitis
F	Hb SS	30	UNK
<b>F</b>	<b>Hb SS</b>	<b>37</b>	<b>1a) Acute respiratory distress syndrome, 1b) Venous occlusive disease secondary to sickle cell disease, 2) Diabetes Mellitus, Renal failure</b>
M	Hb SS	38	UNK
<b>F</b>	<b>Hb SS</b>	<b>39</b>	<b>1a) End-stage renal failure 1b) sickle cell disease</b>
<b>M</b>	<b>Hb SS</b>	<b>40</b>	<b>1a) End-stage renal failure 1b) sickle cell disease</b>
<b>M</b>	<b>Hb SS</b>	<b>41</b>	<b>1a) Multi-organ failure, 1b) Sickle cell disease</b>
<b>F</b>	<b>Hb SS</b>	<b>42</b>	<b>1a) Bronchopneumonia, 1b) Acute chest syndrome, 1c) Sickle cell disease, 2) Pulmonary hypertension</b>
F	Hb SS	43	1a) Pneumonia, 1b) Cerebral haemorrhage/infarction (operated)
<b>F</b>	<b>Hb SS</b>	<b>44</b>	<b>1a) Multi-organ failure, 1b) Mesenteric vein thrombosis, 1c) Sickle cell disease</b>
M	Hb SS	44	UNK
F	Hb SS	45	1a) Congestive cardiac failure, 1b) Left ventricular hypertrophy, dilatation, 2) Sickle cell disease
<b>F</b>	<b>Hb SS</b>	<b>47</b>	<b>1a) End-stage renal failure, 1b) Sickle cell anaemia, 2) Calciphylaxis</b>
M	Hb SS	47	UNK
M	Hb SS	48	1a) Bronchopneumonia, 2) Left ventricular hypertrophy, infarction of liver, spleen and kidneys, urinary tract infection, Sickle cell disease
M	Hb SS	50	UNK
<b>M</b>	<b>Hb SS</b>	<b>51</b>	<b>1a) End-stage liver disease, 1b) Sickle Cell Disease</b>
<b>F</b>	<b>Hb SS</b>	<b>52</b>	<b>1a) Heart failure, 1b) Severe anaemia, 1c) Sickle cell disease; 2) Hypertension, CKD, non obstructive hypertrophic cardiomyopathy</b>
F	Hb SS	58	1a) Septic shock, 1b) Hip abscess, 2) Sickle cell anaemia
<b>M</b>	<b>Hb SS</b>	<b>67</b>	<b>1a) Right sided heart failure, 1b) Pulmonary hypertension, 1c) Sickle cell disease</b>

### **3.5 Discussion**

We have described the prevalence and incidence of end-organ complications in an adult cohort of patients with SCD in a well-resourced urban setting. We have demonstrated that despite the underlying genetic simplicity of the causative mutation that SCA remains a phenotypically diverse condition which still results in an early death for the majority of patients. Longitudinal data from patients with SCD is difficult to gather, partly due to the transitory nature of the patient group, and this study would not have been possible without the sickle cell database.

We have shown significant morbidity in our patient cohort with almost 66% of patients with SCA having at least one complication by the age of fifty. The most common of these were gallstones, retinopathy and microalbuminuria. Despite being a milder phenotype, almost 50% of HbSC patients will have at least one complication by the age of 50. This emphasises the increased health burdens that will be associated with an aging population of patients with SCD.

Although there are limitations to our study design; primarily the inconsistencies inherent in acquiring clinical data both retrospectively and prospectively our data provides an important description of an adult cohort of patients with SCD in an inner city environment in the United Kingdom in the early 21<sup>st</sup> century.

## **Chapter 4 Leukocyte Telomere Length in Sickle Cell Disease**

### **4.1 Introduction**

The consequences of the polymerisation of abnormal HbS have been subdivided into two major processes; namely vaso-occlusion and haemolytic anaemia. Vaso-occlusion results from the dynamic interplay of multiple factors including red cell/vascular endothelial interactions (Hebbel 1997) causing white blood cells (WBC) and red blood cells (RBCs) to become trapped in the microcirculation. This leads to episodic vascular obstruction and down-stream tissue infarction followed by reperfusion injury, resulting in a chronic inflammatory state. Haemolysis of the sickled RBCs leads to the release of free plasma haemoglobin, consumption of nitric oxide (NO) and the generation of free radical species. End-organ damage is a consequence of the tissue damage secondary to ischaemia from vaso-occlusion and vasculopathy secondary to haemolysis. The end result of both pathologies is increased cell death, leading to premature onset of end-organ damage.

Shortened telomere length has been found in DNA extracted from bone marrow and peripheral white blood cells (WBCs) in patients with inherited conditions associated with premature cellular aging (Tchirkov and Lansdorp 2003, Uziel, *et al* 2008, Vulliamy, *et al* 2004, Vulliamy, *et al* 2001b). Short peripheral blood leukocyte telomeres have also been associated with acquired diseases such as cancer (Hosgood, *et al* 2009, McKay, *et al* 2008, Rafnar, *et al* 2009) and heart disease (Brouillette, *et al* 2007, Fitzpatrick, *et al* 2007, Satoh, *et al* 2009) especially those associated with oxidative damage caused by smoking (Brandl, *et al* 2011, Effros 2011, Wafra, *et al* 2011). The chronic inflammatory state and oxidative damage from reduced availability of NO found in SCD may have an effect on telomere length, with the presumption being that shorter telomeres would be found in patients with a more haemolytic phenotype. If so, telomere length could therefore be used as a marker of disease severity.

#### **4.1.1 Telomere Structure and Function**

Telomeres are DNA protein structures that protect chromosome ends from degradation and fusion (Blackburn 2010) and are therefore essential to maintain the integrity of the genome.



The telomeric DNA sequence in humans consists of a sequence of tandem repeats of the six-nucleotide sequence TTAGGG (Moyzis, *et al* 1988) (see Figure 8). These repeats extend for many thousands of bases at the ends of chromosomes and are bound by a highly specialised group of six proteins (the shelterin complex). Shelterin acts as a structure to “cap” ends analogous to the aglet of a shoe lace and includes telomere repeat binding factor 1 (TRF1), TRF2, repressor/activator protein 1 (RAP1), TRF1-interacting protein 2 (TIN2), TIN2-interacting protein 1 (TPP1) and protection of telomeres 1 (POT1). TRF1 and TRF2 are linked to suppression of DNA damage repair pathways. This prevents recognition of the single strand/double strand telomeric tip as damaged. TRF1 also monitors telomere length and TRF2 stabilises the telomeric loop and acts as a protein hub (Xin, *et al* 2008). POT1 maintains telomere integrity and TPP1 interacts with telomerase and is involved in assembly of the shelterin complex (Wang, *et al* 2007) as is TIN2 (O'Connor, *et al* 2006). RAP1 interacts with TRF2 and inhibition of RAP1 leads to increased telomere length (Li and de Lange 2003). The components of the shelterin complex interact with other proteins important to telomerase maintenance including TRF1-interacting ankyrin-related ADP-ribose polymerase (tankyrase, encoded by the *TNKS* gene).

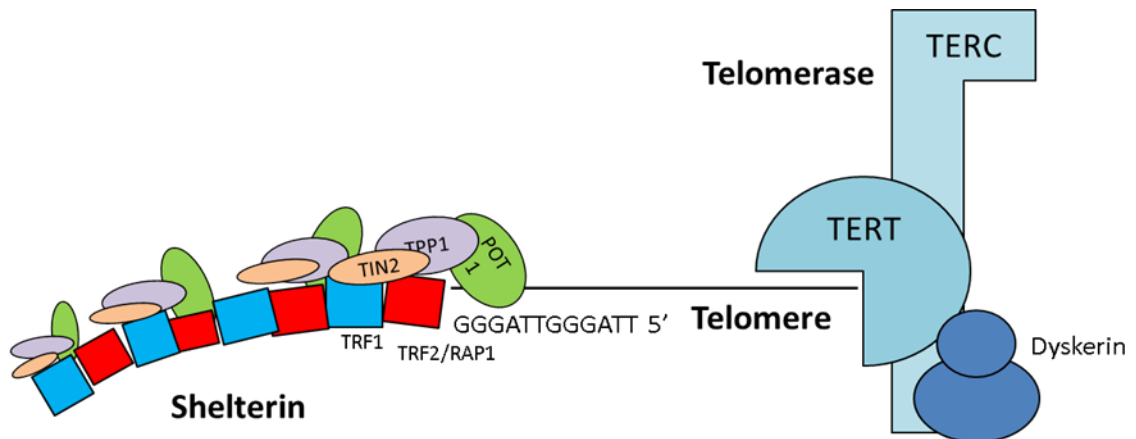


Figure 8: The structure of the human telomere and telomerase complex adapted from *Dokal, ASH Education Book*, December 10, 2011.

In combination, the telomere and shelterin components protect the chromosome end from degradation during replication. Degradation occurs because DNA polymerases only move in the 5' to 3' direction. This means that on the lagging strand (3' to 5') the replication occurs in short pieces (called Okazaki fragments) initiated by RNA primers which are then joined together by DNA polymerase, RNA nuclease, and DNA ligase which convert the RNA primers to DNA and therefore "bridge the gaps". This process requires a second "following" DNA fragment with which to form a connection; at the end of chromosomes this is not present, therefore a section of chromosome (telomere) is lost with each replication. Telomeres therefore prevent the loss of encoding DNA and shorten with increasing cell age. There is however a difference between the estimated loss per division and actual telomere shortening rates. *In vitro* studies have shown that telomeres are highly susceptible to oxidative stress (Serra, *et al* 2000, Von Zglinicki 2000). Telomere shortening due to free radicals explains this difference between the estimated loss per division (approximately 20 base pairs) and actual telomere shortening rates (50-100 base pairs), and thus has a greater potential absolute impact on telomere length than shortening caused by the end-replication problem.

This shortening with increasing cell age also leads to telomeres playing a role in cell senescence. Although telomeres are elongated by telomerase the telomere eventually reaches a critical length leading to failure of function of the shelterin complex. This then causes cells to undergo “crisis”, characterised by chromosomal rearrangements and genomic instability. This process requires the activation of the tumour suppressor protein 53 (p53) pathway (May and May 1999), leading to cell death. The second mechanism of senescence is via the retinoblastoma protein (pRB) which also has a tumour suppressant action (Campisi 2005). Interestingly, telomere length appears to be a heritable trait, however it is unclear whether the inheritance of a shortened telomere predisposes to the conditions associated with shortened telomeres, such as atherosclerosis (Aviv 2012).

#### **4.1.2 Maintenance of Telomeres: Telomerase Structure and Function**

Telomerase is a specialised DNA polymerase that synthesises new telomeric sequences onto chromosome ends (Greider and Blackburn 1985, Greider and Blackburn 1987, Greider and Blackburn 1989). Telomerase consists of two functional parts; Telomere reverse transcriptase, (TERT) and an RNA component (TERC) which provides the template for telomere synthesis) (see Figure 8). However TERT and TERC require additional proteins for regulation and assembly, the best characterised of which is dyskerin, widely studied due to mutations in the encoding gene resulting in the X-linked form of dyskeratosis congenita (DC). TERT expression is very low in the majority of human cell types. However in cellular compartments that require extensive cell division, including stem cells, cancer cells and some white blood cells, TERT is expressed at higher levels and telomere shortening is partially or fully prevented (Counter, *et al* 1995).

Telomerase adds telomere repeats during the S phase and into M phase in the cell-cycle (Diede and Gottschling 1999, Marcand, *et al* 2000). Telomerase only elongates a subset of telomeres in each cell cycle, preferentially lengthening the shortest telomeres (Teixeira, *et al* 2004). Telomere extension is controlled predominantly by the amount of telomerase present, however even in cells with high telomerase activity (such as haematopoietic stem cells), telomeres still shorten with each cell division and with increasing age (Vaziri, *et al* 1994). There are three potential explanations behind this tight regulation of telomerase levels. Firstly, short telomeres

protect against the development of malignancy in long lived organisms. This is supported by the fact that telomerase activity is increased in the majority of human cancers (Kim, *et al* 1994). Secondly the limitation of telomerase levels may prevent competition with alternative DNA repair mechanisms at double-strand-break sites, thus preventing telomeric DNA addition to inappropriate regions. Finally evolutionary selection for short telomeres limits organism life span resulting in a constant influx of varied genetic material into the gene pool.

#### **4.1.3 Telomeres and Disease**

Both short and long telomeres have been linked to disease. The dysfunction of telomeres is thought to be secondary to their shortening to the degree where the shelterin complex is no longer functional. Loss of shelterin function leads to the initiation of the “crisis” referred to above, resulting in apoptosis or senescence. Shortened telomeres secondary to underlying genetic conditions result in similar responses within the cell, however the resultant disease processes are diverse. The most dramatic changes are seen in high-turnover tissues (Herrera, *et al* 1999, Lee, *et al* 1998) but recent research has shown that changes can also occur in low-turnover tissues (Alder, *et al* 2011, Sahin, *et al* 2011). Many of the initial discoveries with regard to the function and maintenance of telomere length came about through the study of a group of monogenic telomere disorders which manifest as a collection of complications, even though symptoms relating to a single organ usually predominate. These clinical findings can be found to varying degrees in all telomere syndromes, whatever the underlying mutation.

##### **4.1.3.1 Monogenic Telomere Disorders**

The best characterised of the monogenic telomere disorders is Dyskeratois congenita (DC). It is defined by the presence of specific muco-cutaneous features (skin hyperpigmentation, oral leukoplakia and nail dystrophy), but includes other additional complications such as bone marrow failure, pulmonary fibrosis and cancer (de la Fuente and Dokal 2007). At a molecular level DC is characterised by the short telomere defect (Alter, *et al* 2007, Mitchell, *et al* 1999, Vulliamy, *et al* 2001a), however the underlying genetic basis and mode of inheritance is varied, with X-linked recessive, autosomal dominant, and autosomal recessive cases all being described (de la Fuente and Dokal 2007, Heiss, *et al* 1998). Figure 9 shows the locations of the

mutations causing DC and the associated syndromes in more detail. However approximately 40% of cases remain genetically unclassified. Other inherited telomere disorders include idiopathic pulmonary fibrosis and aplastic anaemia (AA) (Armanios 2012a, Yamaguchi, *et al* 2003). Both conditions (in contrast to DC) present in adulthood, and are far more common. Idiopathic pulmonary fibrosis is the most prevalent condition ascribed to mutant telomere genes (Armanios 2012b). Liver cirrhosis (a known complication of DC) can also be the first adult presentation of telomerase gene mutations (Calado, *et al* 2009) .

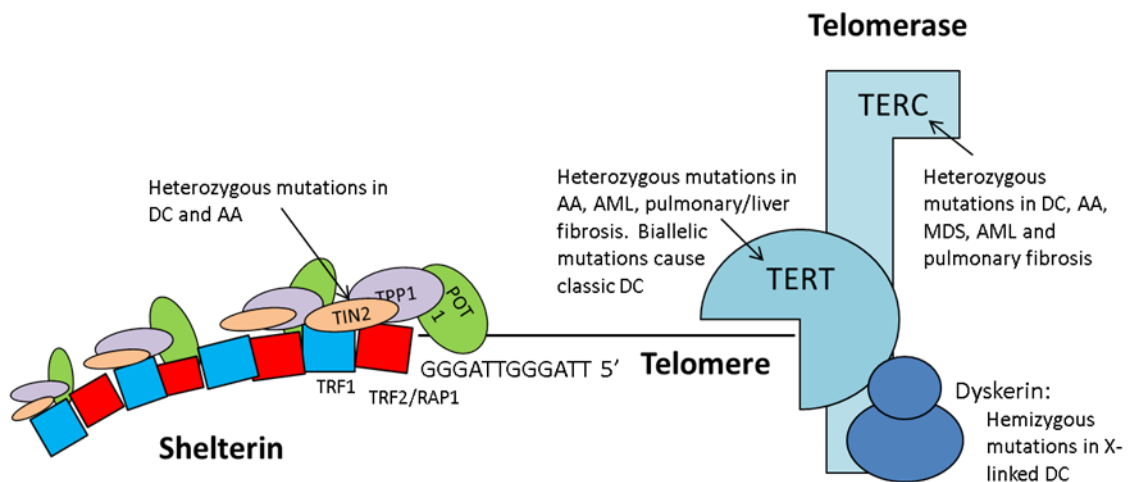


Figure 9: The structure of the human telomere and telomerase complex showing locations of the mutations causing dyskeratosis congenital (DC) and the associated syndromes. AA = aplastic anaemia, MDS = myelodysplastic syndrome, AML = acute myeloid leukaemia.

Adapted from *Dokal, ASH Education Book* ,December 10, 2011

#### 4.1.3.2 Telomere Length and Cancer

Patients with the above telomere syndromes are prone to malignancy. In classical DC the risk is 11 fold compared to the general population (Alter, *et al* 2009) and patients are more likely to get cancers related to high-turnover tissues such as skin and oesophageal tracts. Haematological malignancies are also a complication in both DC and AA patients, commonly presenting as acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS) (Alter, *et al* 2009, Kirwan, *et al* 2009). Telomere shortening limits the long-term proliferative capacity of all cells including those found in the immune system, potentially leading to an acquired immune deficiency, resulting in a failure of cancer surveillance. The alternative hypothesis is that shorter telomeres may lead to genomic instability and therefore an increased risk of cancer (Artandi and DePinho 2010).

Longer telomeres have been found in human cancers, and appear to confer immortality on the abnormal clone leading to uncontrolled proliferation. This most commonly occurs due to the abnormal activation of telomerase (Colgin, *et al* 2003, Colgin and Reddel 1999) but the Alternative Lengthening of Telomeres (ALT) pathway has also been postulated (Bryan, *et al* 1997). This mechanism is not fully understood but recombination events are thought to play a role in telomere maintenance in this method (Dunham, *et al* 2000).

#### **4.1.4 Telomeres and Sickle Cell Disease**

This is the first published study of telomere length in sickle cell disease.

##### **4.1.4.1 Effect of Haemolysis**

As mentioned above (4.1.1) oxidative stress appears to play a key role in telomere shortening. The red blood cells of patients with SCD are constantly undergoing haemolysis resulting in the release of cell free plasma Hb. This in turn leads to consumption and reduction in available NO and generation of free radicals; an oxidative environment could predispose to shorter telomeres.

##### **4.1.4.2 Effect of Inflammatory Environment**

In contrast, chronic inflammation could be associated with longer telomeres and up-regulation of telomerase. Londoño-Vallejo recently presented data on telomere length in idiopathic pulmonary hypertension (IPH) (Londono-Vallejo 2012), a proliferative disorder of pulmonary artery smooth muscle cells. They found longer telomeres in the pulmonary artery smooth muscle cells in patients suffering from IPH compared to controls. Telomere length was also found to correlate with disease severity, with longer telomeres indicating more severe disease. His group also found that telomerase activity was increased in patients suffering from IPH. They theorise that this may be secondary to an inflammatory stimulus. As a chronic inflammatory state exists in patients with SCD, this could, in contrast to the theory of oxidative stress, predispose to longer telomeres.

#### **4.1.4.3 Work by Other Groups**

As mentioned in 4.1.1 tankyrase plays an important role in telomere length regulation. A genome wide association study (GWAS) in SCD has shown that single nucleotide polymorphisms (SNPs) in the telomere length regulator gene *TNKS* are associated with disease severity in SCD (Sebastiani, *et al* 2010). Despite this, telomere length itself has not yet been examined in SCD and therefore its role in understanding the pathophysiology, and disease severity remains unclear.

## **4.2 Objectives**

Shortened telomere length has been associated with a number of disease states. To improve our understanding of the pathophysiology of SCD I examined the effect of haemolysis and inflammation on telomere length.

- a) To measure relative telomere length in white blood cells in adult patients with SCD and compare with controls**
- b) To correlate relative telomere length with markers of haemolysis and disease severity**
- c) To interpret the above findings with respect to the competing influences of haemolysis and inflammation.**

This work has been published (see appendix) (Drasar, *et al* 2014)

## **4.3 Methods**

### **4.3.1 Patients and control subjects**

The study population included a total of 126 healthy controls and 423 patients of African descent with sickle cell disease (SCD) of mixed genotypes (289 HbSS, 111 HbSC, 16 HbS $\beta^+$  thalassaemia and 7 HbS $\beta^0$  thalassaemia) (see Table 9). The patients were recruited through the specialist clinics in the Haematology outpatient unit (King's College Hospital Local Research Ethics Committee protocol 07/H0606/165).

#### 4.3.2 Measurement of Telomere Length

Genomic DNA was isolated from peripheral blood leukocytes. Telomere length measurement utilised a multiplex quantitative polymerase chain reaction (MMqPCR) technique adapted from the original method as described by Richard Cawthon (Cawthon 2009). This method compares telomere repeat sequence copy number (T) to a single copy gene (S – albumin) copy number in a given sample. Duplicate DNA samples were amplified in parallel in a 20µl reaction in a Rotorgene 6000 PCR system (Corbett life sciences) containing 10ng genomic DNA with Dr Jie Jiang.

Telomere primer sequences:

Telg 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'

Telc 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'.

Albumin primer sequences:

Albu 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG-3'

Albd 5'-GCCCGGCCCCGCGCGCCCGTCCCGCCGAAAAGCATGGTCGCCTGTT-3'

The telomere primers generate a short fixed length product only with only telg being able to prime DNA synthesis along the native telomeric sequence due to a 3' terminal mismatch. The primer design of the single gene is such that the S (single-gene) amplicon melts at a much higher temperature than the T (telomere) amplicon due to the addition of a GC-clamp to the 5' end of the single gene primers. Thus signal was acquired at temperature when T amplicon has completely melted but low enough to keep the S amplicon double-stranded enabling binding of SYBR Green I. For each sample, telomere length was expressed as a telomere to single copy gene ratio (T/S ratio) derived from delta  $C_t$  ( $C_t^{\text{telomere}} / C_t^{\text{single-gene}}$ ) based on the standard curve. The standard curve was prepared using three-fold serial dilutions of genomic DNA and was assayed in triplicate. In order to remove variability between runs three reference samples with known T/S ratios were included as internal controls in each run and the results only approved if the relative T/S ratio fell within a 5% variation.



20 µl reactions were set up containing 10 ng genomic DNA, 800nM of each primer, 0.5 mM of dNTPs, 0.5U TaqGold (Applied Biosystems, UK), 3 mM of MgCl<sub>2</sub>, 0.5x Sybr Green I in DMSO and 2.5µl of PCR buffer (Applied Biosystems, UK). Cycling conditions were adapted from the paper by Cawthon and are shown below in Table 8.

Table 8: Cycling conditions for multiplex quantitative polymerase chain reaction (MMqPCR) technique for amplification and measurement of single gene (albumin) and telomere copy number

Stage	Temperature (°C)	Time		
1	95	15 min		
2	94	15 sec		2 cycles
	49	15 sec		
3	94	15 sec		32 cycles
	62	10 sec		
	74	15 sec	Signal acquisition	
	84	10 sec		
	88	15 sec	Signal acquisition	
4	Cool to 72			
	Increase 0.5/step	30 sec/step	Signal acquisition	
	Until to 95			

After cycling was completed data was analysed using Rotorgene 6000 series software version 1.7.

#### 4.3.3 Data Management and Statistical Analysis

Laboratory data including haemoglobin (Hb), WBC, neutrophil count, absolute reticulocyte count, nucleated red blood cell count (NRBC count), gamma-glutamyl transpeptidase (GGT), HbF, ferritin, creatinine, lactate dehydrogenase (LDH), bilirubin, aspartate transaminase (AST) and urine albumin creatinine ratio (ACR) were collected from routine blood results, in steady state clinics contemporaneous to the date of DNA collection. Estimated glomerular filtration rates (eGFRs) were calculated using the 4-point Modification of Diet in Renal Disease (MDRD) formula. Patient age at the time of DNA collection was recorded. Clinical data regarding

treatments at time of DNA collection (transfusion programmes, hydroxycarbamide) were collected from the electronic patient records and sickle cell database. Data was manipulated in Excel (Microsoft, Seattle) and SPSS (IBM, New York). Student's t-test or ANOVA was used to compare relative telomere length subdivided by categorical variables. Simple linear regression was used to analyse correlation between telomere length and continuous variables and multivariate linear regression was corrected for age, gender and alpha genotype. Multivariate analysis of laboratory data was also corrected for use of hydroxycarbamide at time of sample collection.

#### **4.4 Results**

Relative telomere length (T/S) was measured in 549 DNA samples derived from the Sickle Cell Gene Bank using multiplex q-PCR. Of the 549 samples, 126 participants were from healthy controls and 429 from participants with sickle cell disease (SCD) of mixed genotypes. The control group consisted of 77 (61%) female and 49 (39%) male. Age ranged from 6 – 86 (mean 45) years. Laboratory variables were only available for the SCD group. Due to group size, the 16 patients with Hb S $\beta^+$  were excluded from genotype sub-group analysis, and Hb S $\beta^0$  thalassaemia were included with Hb SS as sickle cell anaemia (SCA) (Hb S $\beta^0$  thalassaemia patients have a phenotype severity similar to that of Hb SS patients). Demographic data relating to the patients with SCD are outlined in Table 9.

Table 9: Summary of demographic data for Sickle Cell Patients and sub-groups and the effect of age on relative telomere length

	<b>Controls</b>	<b>SCD</b>	<b>SCA (HbSS and Hb S<math>\beta^0</math>)</b>	<b>Hb SC</b>
<b>Number of patients</b>	126	423	296	111
<b>Male: Female (%)</b>	49:77 (39:61)	174:249 (41:59)	125:171 (42:58)	40:71 (37:63)
<b>Mean age (range)</b>	45 (6 to 86)	33 (17 to 81)	32 (17 to 72)	34 (17 to 68)
<b>Sickle genotypes</b>				
Hb SS (%)	N/A	289 (68)	289 (98)	N/A
Hb SC (%)	N/A	111 (26)	N/A	111 (100)
Hb S $\beta^+$ (%)	N/A	16 (4)	N/A	N/A
Hb S $\beta^0$ (%)	N/A	7 (2)	7 (2)	N/A
<b>Alpha-globin genotypes available *</b>		272	185	74
$\alpha\alpha/\alpha\alpha$ and $\alpha\alpha/\alpha\alpha\alpha$ (%)	N/A	175 (65)	115 (62)	52 (71)
$\alpha\alpha/\alpha-$ and $\alpha-/alpha-$ (%)	N/A	91 (33)	66 (36)	21 (28)
$\alpha\alpha/\alpha\alpha\alpha$ (%)	N/A	6 (2)	4 (2)	1 (1)
<b>Mean T/S ratio (range)</b>	1.78 (0.87 to 4.17)	2.38 (0.56 to 4.87)	2.45 (0.62 to 4.87)	2.20 (0.56 to 3.46)
<b>Correlation with age</b>	R = 0.44 p<0.0001	R = 0.33 p<0.0001	R = 0.29 p<0.0001	R=0.32 p=0.0001

\*  $\alpha$ -genotypes only available for a sub-set of each patient group N/A = not applicable

#### 4.4.1 Relative Leukocyte Telomere Lengths in Sickle Cell Disease

Patients with SCD had significantly longer mean relative telomere lengths and larger range of distribution than the control group, potentially indicating a greater variety of influences in the SCD group (see Table 9 and Figure 10). Mean relative telomere length (T/S ratio) was significantly longer in patients with SCA than controls (SCA 2.38 SD $\pm$ 0.60 vs. controls 1.78 SD $\pm$ 0.62 p<0.0001); and patients with Hb SC than controls (Hb SC 2.20 SD $\pm$ 0.57; vs. controls 1.78 SD $\pm$ 0.62 p<0.0001). Relative telomere length was also significantly longer in patients with SCA vs. those with Hb SC (SCA 2.38 SD $\pm$ 0.60; vs. Hb SC 2.20 SD $\pm$ 0.57 p=0.001) (see Figure 10). Patients with Hb SC had significantly shorter T/S ratio than patients with SCA. The

association with sickle genotype persisted when corrected for age, gender and alpha genotype ( $p = 0.001$ ).

Alpha-genotype data was available for 62% of patients with SCA and 74% of patients with Hb SC. The frequency of alpha-thalassaemia trait in each group is shown in Table 9. There was no significant association of alpha-thalassaemia trait with mean telomere length for the whole group or genotype sub-groups.

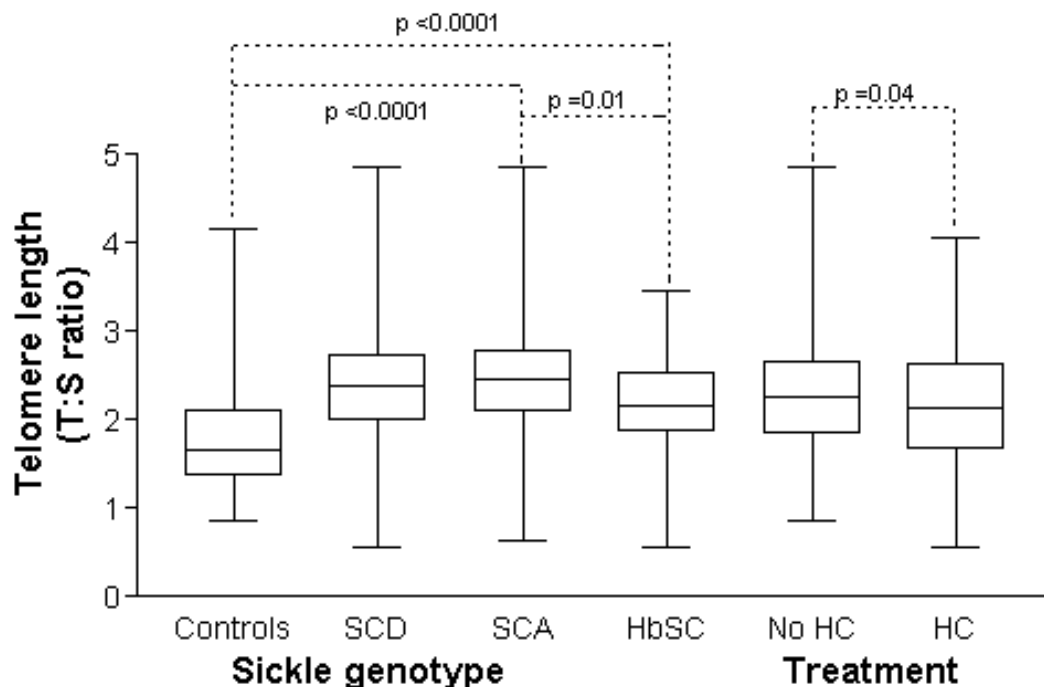


Figure 10: Relative telomere length in patients with sickle cell disease, on or off treatment with hydroxycarbamide.

Controls – Hb AA; SCD – sickle cell disease, SCA – Hb SS and  $S\beta^0$  thalassaemia, Hb SC, No HC refers to patients not on hydroxycarbamide treatment, and HC refers to patients on hydroxycarbamide treatment at the time of DNA collection

#### 4.4.2 Association with Age and Gender

Relative telomere length for the study group and sub-groups was negatively associated with age (see Table 9), as has previously been shown in healthy controls and numerous disease states. This remained significant when the study group was separated into the genotype sub-groups ( $R = 0.44$  and  $p = 0.001$ ) see Figure 11.

There was no significant association between gender and relative telomere length with either simple or multivariate analysis, for the whole group or when separated by sickle genotype.

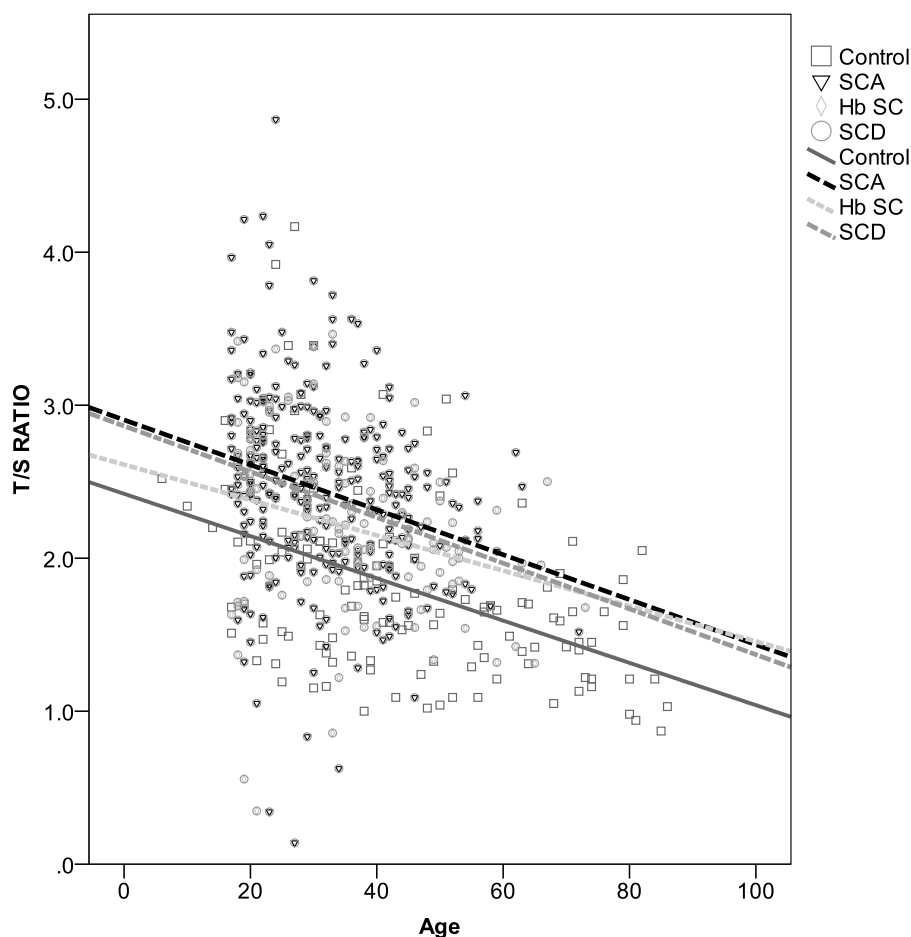


Figure 11: Relative telomere length and its associations with increasing age for study group and sub-groups.

#### 4.4.3 Association with Treatment Modalities

Due to the fact that few patients with Hb SC are treated with transfusion programmes or hydroxycarbamide this analysis was limited to patients with SCA only.

27 patients were on transfusion programmes (simple and exchange) at the time of the sample collection. There was no significant association between telomere length and use of transfusion programme with either simple or multivariate analysis. 108/211 (51%) of the group were untransfused. The remainder had had at least one unit of simple blood transfusion at the time of

sample collection. The range of units transfused were 1-468 with mean 24 units. The amount of simple transfusion was also not associated with telomere length with either simple or multivariate analysis.

57/301 (19%) patients had received hydroxycarbamide treatment for at least 3 months prior to sample collection. Although no association was found between hydroxycarbamide treatment on simple analysis, a significant association was found on multivariate analysis ( $B = -0.300$   $p = 0.04$ ). This shows that patients who were on hydroxycarbamide treatment were found to have significantly shorter telomeres than those without, corrected for age, alpha genotype and gender.

#### 4.4.4 Association with Laboratory Variables

For the simple regression analysis laboratory variables were available for the whole sickle group (407 for the whole group, 296 SCA (Hb SS and Hb S $\beta^0$ ) and 111 Hb SC). The results of this analysis are outlined in Table 10. In the multivariate analysis using linear regression the analysis was corrected for the influence of age, gender, alpha thalassaemia genotype and hydroxycarbamide use. Patients with unknown alpha thalassaemia genotypes were therefore excluded from the analysis. Final analysis included 264 patients for the sickle group analysis, 185 patients with SCA and 74 with Hb SC. The results of this analysis are outlined in Table 11.

Table 10: Correlation of laboratory variables with relative telomere length for patients with SCD and genotype sub-groups. Normally distributed variables were analysed using Pearson's test. Those with skewed distribution were analysed with Spearman's rank test.

Variable	Whole group		SCA		Hb SC	
	Pearson(P) or Spearman (S) value	p value	Pearson(P) or Spearman (S) value	p value	Pearson(P) or Spearman (S) value	p value
<b>Creatinine</b>	<b>-0.22 (S)</b>	<b>&lt;0.0001</b>	<b>-0.21 (S)</b>	<b>&lt;0.0001</b>		N/S
<b>Bilirubin</b>	<b>0.13 (S)</b>	<b>0.008</b>		N/S		N/S
<b>AST</b>		N/S		N/S		N/S
<b>GGT</b>		N/S		N/S		N/S
<b>LDH</b>	<b>0.14 (S)</b>	<b>0.008</b>		N/S		N/S
<b>ACR</b>		N/S		N/S		N/S
<b>Ferritin</b>		N/S		N/S		N/S
<b>WBC</b>	<b>0.20 (P)</b>	<b>&lt;0.0001</b>	<b>0.19 (P)</b>	<b>0.001</b>		N/S
<b>Hb</b>		N/S		N/S		N/S
<b>Platelets</b>	<b>0.15 (P)</b>	<b>0.003</b>	<b>0.11 (P)</b>	<b>0.06</b>		N/S
<b>Neutrophils</b>	<b>0.16 (P)</b>	<b>0.002</b>	<b>0.14 (P)</b>	<b>0.02</b>		N/S
<b>NRBC</b>		N/S		N/S		N/S
<b>Absol. retic</b>	<b>0.12 (P)</b>	<b>0.02</b>		N/S		N/S
<b>HbF%</b>		N/S	<b>-0.13 (S)</b>	<b>0.06</b>		N/S

N/S = no significant result.

Table 11: Multivariate analysis of relative telomere length vs. laboratory variables. All analyses corrected for age, sex, alpha-genotype and hydroxycarbamide use.

Variable	Whole group		SCA		Hb SC	
	R of model	$\beta$ value of variable in model(p value)	R of model	$\beta$ value of variable in model(p value)	R of model	$\beta$ value of variable in model(p value)
<b>Creatinine</b>	<b>0.29</b>	<b>-0.001 (0.06)</b>		N/S		N/S
<b>Bilirubin</b>		N/S		N/S		N/S
<b>AST</b>		N/S		N/S		N/S
<b>GGT</b>		N/S		N/S		N/S
<b>LDH</b>		N/S		N/S		N/S
<b>ACR</b>		N/S		N/S		N/S
<b>Ferritin</b>		N/S		N/S		N/S
<b>WBC</b>	<b>0.31</b>	<b>+ 0.034 (0.005)</b>	<b>0.33</b>	<b>+0.05 (0.003)</b>		N/S
<b>Hb</b>		N/S		N/S		N/S
<b>Platelets</b>		N/S		N/S		N/S
<b>Neutrophils</b>	<b>0.30</b>	<b>+0.03 (0.03)</b>	<b>0.30</b>	<b>+0.03 (0.049)</b>		N/S
<b>NRBC</b>	<b>0.3</b>	<b>+0.03 (0.03)</b>		N/S		N/S
<b>Absol. retic</b>		N/S		N/S		N/S
<b>HbF%</b>		N/S		N/S		N/S

N/S = no significant result

Creatinine initially appeared (after simple regression) to be negatively associated with relative telomere length. However this association disappeared on regression analysis, once the influence of sickle genotype was removed. There was also initial positive association between relative telomere length and markers of haemolysis (LDH, bilirubin and absolute reticulocyte count), however, these did not persist on multivariate analysis. Nucleated red blood cell count (NRBCs), were positively associated with relative telomere length on multivariate analysis alone but this disappeared when corrected for sickle genotype.

The only persistent associations (in simple correlation and multivariate analysis) were found between WBC and neutrophil count and relative telomere length. There were no significant associations for any laboratory variables and telomere length in the Hb SC group in either simple correlation or multivariate analysis, possibly due to Hb SC being a milder disease so that any affects would only become discernible with a larger sample size.

## 4.5 Discussion

We present data from relative telomere measurements from a large, well characterised group of patients with sickle cell disease. qPCR is an increasingly used method of measuring telomere length due to the cost and time implications of the Southern blot technique. We have shown that T/S ratio appears to be extremely diverse in sickle cell disease, with a much larger range of lengths than those seen in our control population. As in all previous studies on telomere length, relative telomere length decreases with increasing age, in all sub-groups analysed. The most striking finding was the significantly longer telomeres found in sickle cell patients compared to our control population. Interestingly, patients with SCA also had significantly longer telomeres than patients with Hb SC. This persisted when corrected for age, gender and alpha-thalassaemia genotype. Although longer telomeres have been found in the malignant setting this finding was surprising given the strong evidence for the role of oxidative damage in telomere shortening, and the knowledge that sickle cell patients (due to chronic haemolysis) are naturally predisposed towards free radical formation (see 1.2).

One possible explanation for this is that we are looking at different cell populations in the two groups. The DNA used was extracted from peripheral blood and would therefore be derived from all circulating nucleated cells (WBCs and potentially NRBCs). NRBCs would be increased in patients with SCD due to high red cell turnover and premature release of erythroid precursors prematurely into the blood. In our cohort the mean NRBC was 5.7 per hundred WBC (range 0.02 – 34.5). However the lack of significant association between the NRBC count and relative telomere length appears to make this unlikely.

The other possible explanation relates to the effect of inflammation on telomere length. As previously discussed (see 4.1.4.2), Londoño-Vallejo recently presented data showing longer telomeres in idiopathic pulmonary hypertension (Londono-Vallejo 2012), theorising that this may be secondary to an inflammatory stimulus causing an up-regulation of telomerase activity. Our initial data appears to bear this out with relative telomere length being positively associated with WBC and neutrophil count in both simple and multivariate analysis. The fact that patients with Hb SC also have relatively shorter telomere lengths than patients with SCA supports the



underlying inflammation hypothesis. This theory is given greater weight as our data also shows that telomere length is shorter in those patients who had been treated with hydroxycarbamide for at least 3 months prior to the sample collection occurring. Hydroxycarbamide is known to have anti-inflammatory effects (via suppression of WBC count and down-regulation of cytokines (Charache, *et al* 1996, Laurance, *et al* 2011)). HbF did not appear to be associated with relative telomere length suggesting that this effect on telomere length is not mediated via Hb F increase. An alternative explanation harks back to HCs original purpose, as a chemotherapeutic agent in acute leukaemia. Its primary mechanism of action is by inhibition of ribonucleotide reductase, thus decreasing the production of deoxyribonucleotides and halting DNA replication. This could therefore also have the effect of preventing the extension of or limiting the length of telomeres in patients undergoing treatment.

There were limitations to our study. Data shows that the rate of telomere attrition is far higher in early life compared to adults (Rufer, *et al* 1999, Sidorov, *et al* 2009) which has a significant effect on adult T/S ratio. Therefore it may be more appropriate to study the length of telomeres in children and young adults with SCD and correlate with biological factors, such as degree of haemolysis, present at that time. It is also possible that the qPCR method has limitations. A recent paper by Aviv *et al* (Aviv, *et al* 2011) has compared the qPCR and Southern blot analysis in two experienced laboratories, including reproducibility. High correlations were seen between the two sets of measurements of T/S ratio highlighting good reproducibility of results “intra-technique”. The measurement error was however 6.45% for the qPCR method compared to 1.74% for Southern blots. This leads to potential problems when looking at inter-individual variation in relation to factors effecting T/S ratio particularly age. Assuming a rate of shortening of T/S ratio of ~30 nt per year, the error of measurement accounts for 13 years by the qPCR method vs. 4 years by the Southern blot method in a patient with a T/S ratio of 6kb. Despite this they feel that a 6% error may be acceptable should the purpose of the data to be used to rank individuals for risk of developing specific complications relative to their age norms.

In conclusion, despite the potential limitations of the technique, patients with SCD do have significantly longer relative leukocyte telomere lengths compared to healthy controls. The relatively small number of patients limited analysis of telomere lengths with organ dysfunction

but sample size was sufficient to show correlation of telomere length with WBC and neutrophils, markers of inflammation. Another limitation is that this cross-sectional study only allows inference of associations. The shorter telomeres in patients treated with HC offers an opportunity to design a prospective longitudinal study to compare telomere lengths before and after HC therapy within the same patient. We did not control for life-style variables such as smoking and body mass index, factors that can affect telomere length.

Nonetheless, given the consistent association of telomere lengths with parameters indicative of inflammatory activity, this study confirms the key role of inflammation underlying the pathophysiology of SCD.

## Chapter 5 The Effect of Duffy Antigen Receptor for Chemokines (DARC) on Severity in Sickle Cell Disease

### 5.1 Introduction

The key-stone of the pathophysiology of sickle cell disease (SCD) remains the polymerisation of abnormal haemoglobin S which sets off a cascade of downstream effects. SCD, despite being a monogenic disorder is remarkable for its diverse presentations and degrees of severity (see 0). Multiple studies have implicated genetic polymorphisms and biomarkers as potential predictors for the development of specific complications and overall disease severity (Thein 2008). Indeed WBC count is known to correlate with specific sickle cell anaemia complications and risk of mortality (Balkaran, *et al* 1992, Platt, *et al* 1994), therefore polymorphisms affecting the WBC count could potentially affect the severity and disease phenotype of SCD. Recent work has shown that people who lack the Duffy antigen on the surface of their red cells have significantly lower neutrophil counts and WBC (Reich, *et al* 2009), and that this explains the phenomenon of “benign ethnic neutropenia” which had been long observed in haematology clinics in people of African descent.

The Duffy blood group was first described in 1950 by Cutbush *et al* (Cutbush and Mollison 1950) following a transfusion reaction in a haemophiliac patient. One year later a second haemolytic transfusion reaction in another patient was described by Ikin *et al* (Ikin, *et al* 1951). It was postulated that two different Duffy antigens ( $Fy^a$  and  $Fy^b$ ) existed encoded by two genes ie  $Fy^a$  and  $Fy^b$ , the transfusion reactions having been caused by antibodies in the patients' serum reacting with the antigen on the surface of the donor red blood cell. Cutbush and Mollison carried out family studies and showed that expression of the Duffy antigens was inherited in a dominant manner (Cutbush and Mollison 1950). It was also noted that different ethnic populations appeared to have differing frequencies of expression of the Duffy antigen and that “estimates of the incidence of the  $Fy^a$  gene in various populations may prove valuable in anthropological studies” (Cutbush and Mollison 1950).

The Duffy antigen consists of a glycosylated membrane protein. Four antigen phenotypes exist corresponding to protein expression on the surface of cell membranes; Fy(a+b+), Fy(a+b-), Fy(a-b+), which are all considered to be Duffy positive, and Fy(a-b-) which is referred to as Duffy negative (also called Duffy null). The Duffy negative phenotype has been found to be particularly prevalent in people of African heritage (Howes, *et al* 2011, Sanger, *et al* 1955) (see Figure 12). However in this group the Duffy antigen (Fy<sup>b</sup>) is still present on the surface of non-erythroid cells and is only absent on the red blood cell (RBC), so called “erythroid silent”. A different polymorphism causes the Duffy negative phenotype in Caucasian populations. In this group the Fy antigen is missing on both erythroid and non-erythroid cells. The erythroid silent form is thought to have persisted from selection pressure of *P. vivax* and *P. knowlesi*, now no longer prevalent in sub-saharan Africa. The Duffy antigen has been shown to be the erythroid receptor for *P. vivax* and *P.knowlesi*, as without the presence of the Duffy antigen the malaria parasites are unable to enter the red blood cell to continue their lifecycle (Barnwell, *et al* 1989, King, *et al* 2011). *P. vivax* and *P.knowlesi* are rarely found in areas of the world with high incidence of Fy(a-b-) (Howes, *et al* 2011, Miller, *et al* 1976).

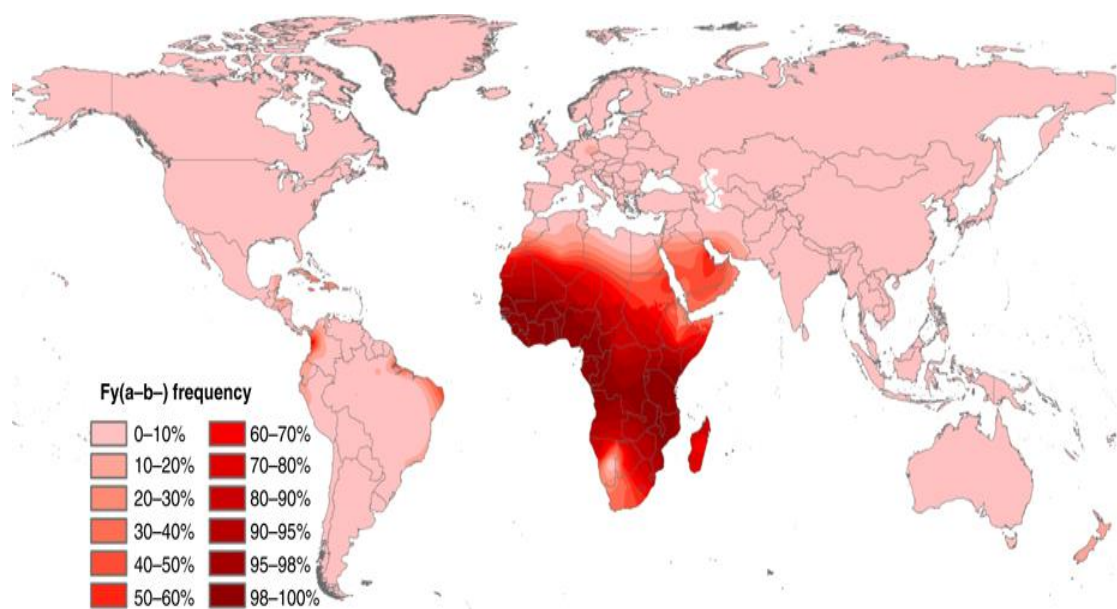


Figure 12: Global distribution and prevalence of the Duffy negative phenotype. ( From (Howes, *et al* 2011))

The common Duffy phenotypes result from 10 possible genotypes from the 4 alleles (although rare variants also exist) as shown in Table 11. The *FY* locus is located on the long arm of chromosome 1 (1q22 to q23 (Mathew, *et al* 1994)) and consists of co-dominant alleles *FY*\*A and *FY*\*B differentiated by a single base substitution (G125A) (see Figure 12).

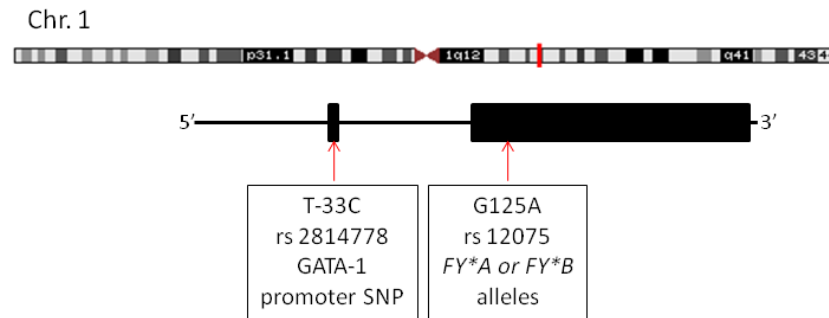


Figure 13: Structure of the *FY* locus indicating the SNP associated with *FY*\*A and *FY*\*B alleles and the GATA-1 promoter SNP causing *FY*\*B<sup>ES</sup> and *FY*\*A<sup>ES</sup> (erythroid silent phenotype)

The Fy(a-b-) phenotype found in people of African heritage is caused by a T to C substitution in the gene promoter at nucleotide -33. The SNP substitution disrupts binding to the GATA-1 and prevents transcription resulting in the null “erythrocyte silent” (ES) phenotype found in this group. This variant is most commonly associated with the *FY*\*B allele (resulting in *FY*\*B<sup>ES</sup>) but can also occur with the *FY*\*A allele (resulting in *FY*\*A<sup>ES</sup>).

Table 12: Common Duffy phenotypes and genotypes with world-wide genotype frequency. (Adapted from (Howes, *et al* 2011))

Phenotype	Genotype	Genotype frequency (%)			
		Africa	Americas	East Asia	Europe
Fy(a+b+)	<i>FY</i> *A/*B	5	32	<1	52
Fy(a+b-)	<i>FY</i> *A/*A <sup>ES</sup>	0	0	3	0
	<i>FY</i> *A/*A	2	18	97	13
	<i>FY</i> *A/*B <sup>ES</sup>	13	10	0	0
Fy(a-b+)	<i>FY</i> *A <sup>ES</sup> /*B	0	0	0	0
	<i>FY</i> *B/*B	3	20	<1	35
	<i>FY</i> *B/*B <sup>ES</sup>	11	10	<1	<1
Fy (a-b-)	<i>FY</i> *A <sup>ES</sup> /*A <sup>ES</sup>	0	0	0	0
	<i>FY</i> *A <sup>ES</sup> /*B <sup>ES</sup>	1	0	0	0
	<i>FY</i> *B <sup>ES</sup> /*B <sup>ES</sup>	65	10	0	<1

ES = erythroid silent

### **5.1.1 Function of the Duffy Glycoprotein**

The Duffy glycoprotein can bind a variety of chemokines and is hence also referred to as the Duffy antigen receptor for chemokines (DARC). DARC acts as a non-specific chemokine receptor for a number of pro-inflammatory cytokines including IL8, melanoma growth-stimulating activity and monocyte chemoattractant protein 1 (Chaudhuri, *et al* 1994, Neote, *et al* 1994). In vitro and in vivo work suggests that the red blood cell DARC acts in two main ways a) to prevent WBC activation in the systemic circulation and b) to stop chemokine dissemination from the blood into the organs (Darbonne, *et al* 1991, Rot 2005). As such, DARC expression could provide protection from excessive chemokine release as well as limiting the activation of WBCs.

### **5.1.2 Relevance to Sickle Cell Disease**

The role of the Duffy protein, along with its variable expression in the population has potential for impact on the severity and pathophysiology of sickle cell disease. Two pathways have been postulated (Durpes, *et al* 2010, Platt, *et al* 1994) that convey an effect of Duffy phenotype (presence or absence of DARC) on the clinical severity of sickle cell disease. Interestingly, one associates lack of DARC (with a lower WBC) with potentially milder disease, the other with potentially more severe disease (relating to its role as a receptor for chemokines). The suggested explanations are: 1) Lack of DARC could be associated with a more severe clinical course as inflammatory cytokines would have longer circulatory half-lives in Duffy negative patients, potentially resulting in increased oxidative damage. 2) Lack of DARC is associated with benign ethnic neutropenia and a lower WBC count. This condition results in a lower baseline WBC, but no apparent effect on response to infection, with normal cellularity and maturation of all cell lines on examination of the bone marrow, with no evidence of myelodysplasia. The precise mechanism is unknown but may relate to the Duffy antigen's interactions with neutrophil activating peptide-2, deficiencies of which lead to neutrophils being abnormally retained in the bone marrow. Potentially an absence of the Duffy antigen could lead to a similar situation with neutrophils not migrating out into the peripheral blood. WBC count is known to independently influence the severity of SCD, possibly due to the role of the WBC in potentiating vaso-occlusive episodes. Platt *et al* found that patients with sickle cell anaemia who had an elevated white-cell count (15,100 per cubic millimetre) had a higher risk of death (2.2 vs. 1.2 per 100 person-years) (Platt, *et al* 1994).

It is also important to note the potential effect of ethnic stratification on the prevalence of the Duffy positive phenotype in the SCD population. As the Duffy null genotype is a marker of African origin, the presence of Duffy antigen infers admixture with a Caucasian population, and thus potentially the inheritance of other polymorphisms across the genome.

### **5.1.3 Previous Work by Other Groups**

Other groups have examined the relationships between Duffy phenotype and markers of disease severity or the postulated disease phenotypes in SCD but no clear consensus has been reached. This could be due to the different populations from which each of the studies are drawn (with different degrees of non-African admixture); however, the proportions of Duffy positive patients are similar in at least 3 of the groups. The retrospective nature of the work has also led to incomplete datasets. Each group has also looked at different markers of disease severity, including scoring systems unique to the institution (Schnog, *et al* 2000) and a different range of clinical complications. The biological parameters analysed also vary from group to group. Schnog *et al* presented a small cohort of patients, with mixed sickle genotype. Sickle cell genotype was not corrected for during the analysis and this can have far-reaching consequences on all the parameters discussed in the paper. Thus, the conclusions drawn as to the non-significance of Duffy phenotype should not be taken on face value. This is also the only study to report non-significance of the effect of Duffy phenotype on WBC count. My discussion is therefore focused on the other three studies. The results of all four published studies are summarised in Table 13, which for brevity only includes significant findings.

The three studies focus on patients with Hb SS or Hb S $\beta^0$  and include patients from geographically diverse areas of the world. None of the groups have corrected for alpha-thalassaemia or HbF (the 2 well characterised modifiers of disease severity) in their analyses. The genders were equally divided in all the groups. The Brazilian population is clearly the most admixed with 75% of the patients being Duffy positive. This raises the possibility of ethnic stratification and confounding by other, “more Caucasian” polymorphisms as this is clearly a very different population from the other 2 studied. The mean age of the three groups was 30-35 years. Only Afenyi-Annan *et al* performed multivariate analysis correcting for age and gender.

Table 13: Summary of the significant associations with markers of disease severity in SCD and Duffy phenotype

Patient characteristics	Schnog et al (Schnog, et al 2000)	Afenyi-Annan et al (Afenyi-Annan, et al 2008)	Nebor et al (Nebor, et al 2010)	Mecabo et al (Mecabo, et al 2010)
Population location	Curaçao	African-American	Guadeloupe	Brazil
Number (Genotype)	30 (Hb SS) 21 (Hb SC)	237 (Hb SS)	212 (Hb SS)	87 (SCA)
Number Duffy +ve (%)	15 (29)	63 (27)	49 (23)	65 (75)
Number Duffy -ve (%)	36 (71)	174 (73)	163 (77)	22 (25)
Stroke	N/S	N/S	Not done	Increased incidence of stroke in Duffy -ve <b>p = 0.008</b>
Priapism	Not done	Not done	N/S	Increased incidence of priapism in Duffy -ve <b>p = 0.02</b>
Pulmonary hypertension*	Not done	N/S	Not done	Increased incidence of PHT in Duffy -ve <b>p = 0.009</b>
Number of organs affected	Not done	Increased number of organs affected in Duffy -ve <b>p = 0.002</b>	Not done	Not done
Hb (g/dL)	N/S	N/S	N/S	Lower Hb in Duffy -ve <b>p = 0.04</b>
WBC (x10 <sup>9</sup> /L)	N/S	Lower WBC in Duffy -ve <b>p = 0.02</b>	Lower WBC in Duffy -ve <b>p = 0.007</b>	Not done
Neutrophils (x10 <sup>9</sup> /L)	N/S	Not done	Lower Neuts in Duffy -ve <b>p = 0.002</b>	Not done
eGFR (ml/min/1.73m <sup>2</sup> )	Not done	N/S	N/S	Not done
Albumin:Creatinine ratio	Not done	Increased risk of proteinuria in Duffy -ve <b>p = 0.002 (urine dip +ve)</b>	N/S	Not done
LDH (IU/L)	Not done	Not done	Not done	Higher LDH in Duffy -ve <b>p = 0.009</b>

\*Pulmonary hypertension (PHT) was defined as a tricuspid regurgitant jet velocity equal to or above 2.5 m/s

\*\* Proteinuria defined as urine dip >1+

N/S = non-significant result



A broad spectrum of end-organ complications is covered by all groups. Although no significant association was found with specific clinical complications, Afenyi-Annan et al reported an overall increase in the number of organs affected in the Duffy negative group. Mecabo et al also found that the Duffy negative phenotype appeared more severe, but with an increased risk of complications associated with the so-called “haemolytic phenotype”. The corollary here is that the Brazil group have a very different distribution of Duffy phenotypes to the other group, which may confound their conclusions, particularly in view of not correcting for age, alpha-thalassaemia or HbF levels.

All three groups examined the effect of Duffy phenotype on common haematological and biological parameters. Afenyi-Annan et al and Nebor et al both found WBC and neutrophil count to be lower in Duffy negative patients, in agreement with previously published data (Reich, *et al* 2009). The American group also found a significant association with Duffy negative phenotype and the presence of proteinuria as measured on urine dip stick. Mecabo et al found that Duffy negative phenotype was significantly associated, not only with the end organ complications associated with the haemolytic phenotype, but also with markers of haemolysis themselves.

In summary, the negative results of Duffy effect on disease severity by Schnog et al can be put to one side on the basis of small sample size and a mixed sickle genotype cohort. However, the findings of the other groups also need re-examining with a further sample set, particularly in view of the admixed nature of the Brazilian cohort who presented the most significant positive findings.

#### **5.1.4 Definitions of Severity**

The definition of disease severity in SCD is rendered difficult by the sheer diversity of clinical complications and range of biological variables found even within patients of the same sickle genotype. Attempts have been made to associate specific complications with pathological sub-phenotypes (haemolytic and vaso-occlusive) but patients often present with features and

complications of both. As shown above there is no current consensus on definition of “severe disease” although steps have been made towards definition of end-organ damage secondary to SCD (Ballas, *et al* 2010).

In our study we aim to combine association of Duffy phenotype with clinical markers (end organ damage and hospital admission data) markers of disease severity and laboratory markers of potential pathological phenotype in a single cohort.

## **5.2 Objectives**

White blood count is known to be a modifier of disease severity in SCD. Benign ethnic neutropenia has been shown to be caused by homozygous inheritance of the Duffy null phenotype. The objectives for this chapter are to:

- a) Characterise the DARC genotypes of the King’s College Hospital Cohort and derive predicted Duffy phenotype**
  
- b) To look for correlations between Duffy phenotype and markers of clinical severity including number of acute admissions, length of stay, time to readmission and presence of end-organ damage**
  
- c) To correlate Duffy phenotype with biological variables including white blood cell count and markers of haemolysis**

This data has been published (see appendix) (Drasar, *et al* 2013)

## 5.3 Methods

### 5.3.1 Genotyping

DNA was extracted from buffy coats obtained via the sickle cell gene bank (REC 07/H0606/165) and prepared using standard methods. Only the *DARC* promoter polymorphism (C/T at -33) was genotyped. Presence or absence of this polymorphism was ascertained using a Taq Man allelic discrimination assay from ABI biosystems) (see 6.3.2.1). Individuals with genotypes T/T or C/T were assumed to have Duffy positive phenotypes whereas those with genotype C/C were assumed to be Duffy negative.

### 5.3.2 Data Management and Statistical Analysis

Biological data including haemoglobin (Hb), lactate dehydrogenase (LDH), WBC, neutrophil count, HbF, reticulocyte count, ferritin, creatinine, urine albumin creatinine ratio (ACR), cystatin C and erythropoietin levels were collected from routine blood results in steady state clinic during a 2 year period from January 1<sup>st</sup> 2009 to December 31<sup>st</sup> 2010. Estimated glomerular filtration rates (eGFRs) were calculated using the 4-point Modification of Diet in Renal Disease (MDRD) formula.

Clinical data including the development of specific complications (stroke, priapism, leg ulcers, acute chest syndrome, avascular necrosis, retinopathy, tricuspid regurgitant jet velocity  $\geq 2.5$ m/s and gallstones) were collected from the electronic patient records and sickle cell database. Admission data were also collected for the 2 year study period including length of stay, time to readmission and number of admissions. Patient age at the end of the study period was recorded. Variables were log transformed where appropriate to obtain a normal distribution. Data was manipulated in Excel (Microsoft, Seattle) and SPSS (IBM, New York). For admission data groups were compared using the Student's t-test or linear regression for multivariate analysis. For clinical complication data groups were compared using the student's t-test or binomial logistic regression for multivariate analysis. The regression analysis for biological variables was performed in Stata 11 (Stata Corp) by Dr Tony Fulford (London School of Hygiene and Tropical Medicine, London). This analysis pooled all data from each individual allowing for dependency between multiple observations on the same individual using random

effects models fitted either using generalised least squares or, when the dependent variable was either binary (logistic regression) or censored (interval regression), by maximum likelihood.

## 5.4 Results

The DARC promoter polymorphism (DARC -33 C/C) underlying the erythroid silent (Duffy negative phenotype) was determined by TaqMan analysis in 272 patients. 165 (61%) of the group were female and 107 (39%) male. Ages ranged from 17 to 74 years (mean 36 years). Admission data was available on the whole cohort with 112/272 patients having at least one admission during the study period. Complete data for end-organ damage was available in another subset (180/272). The clinical characteristics of the study group (and sub groups) are outlined in Table 14.

Table 14: Summary of demographic data for study group and sub-groups. Laboratory data (sub-group 1) was available on the whole cohort. Sub-group 2 consisted of 112 patients who had at least 1 hospital admission during the 2 year study period. Sub-group 3 was limited to Hb SS and Hb S $\beta^0$  patients who data available on end-organ damage.

	Whole cohort	Sub-group 2: Admitted patients	Sub group 3: End-organ damage
<b>Number of patients</b>	272	112 (41)	180 (66)
<b>Male: Female (%)</b>	107:165 (39:61)	48:64 (57:43)	70:110 (39:61)
<b>Mean age (range)</b>	36 (17 to 74)	34 (18 to 74)	34 (17 to 68)
<b>Sickle genotypes</b>			
Hb SS (%)	174 (64)	89 (79)	174 (97)
Hb SC (%)	80 (30)	17 (15)	excluded
Hb S $\beta^+$ (%)	12 (4)	1 (1)	excluded
Hb S $\beta^0$ (%)	6 (2)	5 (5)	6 (3)
<b>Alpha genotypes*</b>	245 (90)	101 (90)	162 (90)
$\alpha\alpha/\alpha\alpha$ (%)	148 (58)	61 (60)	92 (52)
$\alpha\alpha/\alpha-$ and $\alpha-/ \alpha-$ (%)	82 and 13 (32 and 5)	30 and 8 (30 and 8)	55 and 13 (31 and 7)
$\alpha\alpha/\alpha\alpha$ (%)	2 (<1)	2 (2)	2 (1)

\* alpha genotypes were only available for a sub-population of the cohort and sub-groups

The Duffy phenotype was predicted to be negative based on homozygous inheritance of the polymorphism (DARC -33C/C) in 243 (89%) of patients and positive for heterozygous

inheritance or absence of the polymorphism (DARC -33T/C and -33T/T) in 26 (10%) and 3 (1%) patients respectively, which indicates a degree of admixture with non-African ancestry (Table 15). This is unsurprising as our population is drawn from both African-Caribbean and West African populations. There was no significant difference between the frequencies of the Duffy polymorphisms between the different sickle genotypes. The predicted phenotypes were then used to look for associations with markers of disease severity.

The aim of this study was to investigate whether the predicted Duffy status had an influence on disease severity in SCD using the following parameters. 1) Laboratory variables. Specific laboratory variables are associated with increased incidence of specific complications and poor outcomes (e.g. raised WBC or lower Hb). This information can therefore be used as a marker of disease severity. 2) Admission cohort. Admission to hospital with painful episode (or other sickle cell complications), with frequent attendance or prolonged stay is a well acknowledged marker of severity. 3) End organ damage. Presence of end-organ damage is a significant contributor to morbidity and mortality in sickle cell disease and therefore a marker of disease severity.

Table 15 Duffy genotype and phenotype for study group and sub-groups. Laboratory data (sub-group 1) was available on the whole cohort. Sub-group 2 consisted of 112 patients who had at least 1 hospital admission during the 2 year study period. Sub-group 3 was limited to Hb SS and Hb S $\beta$ 0 patients who data available on end-organ damage.

	Whole cohort	Sub-group 2: Admissions data	Sub group 3: End-organ damage
<b>Duffy genotypes</b>	272	112	180
-46 C/C (%)	243 (89)	97 (87)	163 (91)
-46 C/T (%)	26 (10)	14 (13)	14 (8)
-46 T/T (%)	3 (1)	1 (<1)	2 (1)
<b>Duffy phenotypes</b>	272		
Duffy negative (%)	243 (89)	97 (87)	163 (91)
Duffy positive (%)	29 (11)	15 (13)	17 (9)

Biological variables were available on the entire study group. Logistic regression was used to maximise the power of the dataset using multiple episodes from each patient. Multivariate analysis showed significantly lower WBC and neutrophil counts for Duffy negative patients

( $p = 0.008$  and  $p = 0.001$  respectively) (see Table 16). Interestingly, raised Cystatin C and lower estimated glomerular filtration measurement using the Hoek formula (Hoek, *et al* 2003) were significantly associated with the DARC positive genotype ( $p = 0.002$  and  $0.001$ , respectively), although no association was found with ACR. A summary of all the results is shown in Table 16. There was no change in influence of Duffy on WBC and neutrophil count during the acute phase compared to the steady state.

Table 16: Laboratory values in Duffy positive and Duffy negative patients: Difference between groups is expressed either as a percentage or absolute difference depending on whether the variable was analysed in the log.

Biological variable	Effect of Fy phenotype on variable	% Difference between groups (95% CI)	p value
WBC ( $\times 10^9/L$ )*	Fy +ve higher	14% (4 - 26)	<b>0.008</b>
Neutrophils ( $\times 10^9/L$ )*	Fy +ve higher	23% (9 - 38)	<b>0.001</b>
LDH (IU/L)*	No change	7% (-4 - 19)	0.2
Reticulocyte count ( $\times 10^9/L$ )*	No change	-3% (-11 - 7)	0.6
HbF (%)*	No change	-10 (-39 - 32)	0.6
Ferritin (ng/mL)*	No change	49 (-15 - 125)	0.06
MDRD eGFR (ml/min/1.73m <sup>2</sup> )*	No change	-11.82 (-30.07 - 6.42)	0.2
Cystatin C*	Fy +ve higher	20 (7 - 35)	<b>0.002</b>
Hoek formula eGFR*	Fy +ve lower	-16 (-25 - -7)	<b>0.001</b>
Erythropoietin*	No change	-8 (-25 - 9)	0.3
Albumin:Creatinine ratio*	No change	52 (-18 - 182)	0.2
Biological variable	Fy +ve vs. Fy -ve	Absolute Difference between groups (95% CI)	p value
Hb (g/dL)	No change	-0.20 (-0.67 - 0.28)	N/S

All analyses corrected for age, sex, sickle genotype, alpha genotype and whether sample acute or steady-state. Fy = Duffy phenotype (positive or negative).

\* Variables log transformed prior to inclusion in the analysis

Admission data was available for the whole cohort (272 patients). 112/272 (41%) of the patients had at least 1 admission during the 2 year study period. There were 313 admissions from 112 patients, ranging from 1 to 19 per patient (mean 3). Length of stay ranged from 1 to 74 days (mean 7). 69 patients (61%) were readmitted within the study period (range 3 - 627

days, mean 158). There was no significant difference in frequency of Duffy positive status between the admitted and non-admitted groups in simple or multivariate analysis. Within the admitted group there was no significant difference between Duffy positive and Duffy negative patients for length of stay and the total number of admissions either using simple or multivariate analysis. However the mean time to readmission was significantly longer in the Duffy negative group using simple analysis (Duffy negative mean 168 days SD±151 vs. Duffy positive mean 71 days SD±95)  $p = 0.004$  (see Figure 14). This significance persisted when corrected for patient age, sickle and alpha genotypes and sex ( $R = 0.51$ )  $p = 0.004$ .

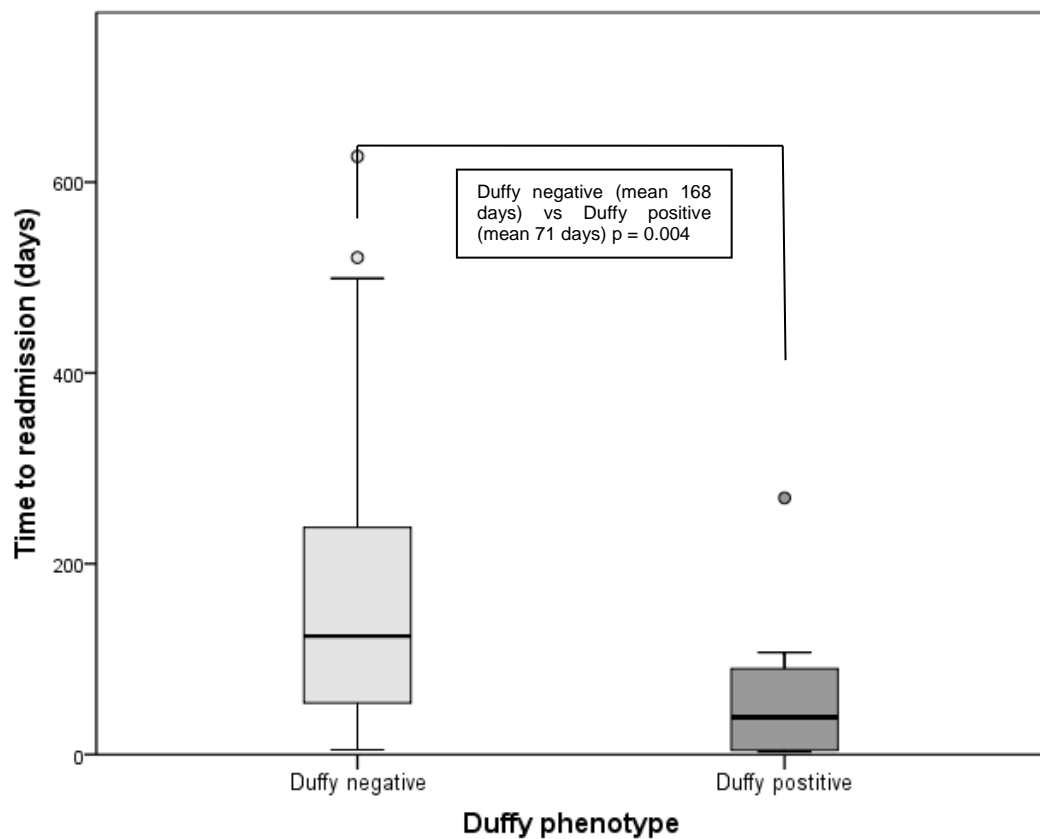


Figure 14: Difference in time to readmission between Duffy positive and Duffy negative patients

Data pertaining to clinical complications were limited to patients with Hb SS and Hb S $\beta^0$  (180/272) as it is well known that Hb SC and Hb S $\beta^+$  genotypes have a milder disease with complications (see Chapter 3). Frequency of clinical complications divided between Duffy phenotypes is shown in Table 17. Using simple analysis there was a significantly higher proportion of Duffy positive patients with leg ulcers ( $\chi^2$  5.21  $p = 0.02$ ) than Duffy negative patients. All other associations were non-significant. This persisted when corrected for age,

sex and alpha thalassaemia genotype (Wald  $\chi^2$  4.67  $p = 0.03$ ). 76/163 (47%) of Duffy negative patients had at least 1 complication versus 13/16 (81%) of Duffy positive patients. Although the difference was significant difference ( $\chi^2$  6.75  $p = 0.02$ ) in the simple analysis, the significance failed to persist in the multivariate analysis (see Table 17) with only age remaining a significant factor influencing incidence of complications.

Table 17: End-organ damage in Duffy positive and Duffy negative SCA patients

	Number of patients with clinical complication (%)		Simple analysis		Binomial regression <sup>\$</sup>	
Clinical complication	Fy negative n = 163	Fy positive n = 13	$\chi^2$ value	p value	Wald	p value
<b>Respiratory complications</b>						
Chest crisis	36 (22)	4 (24)	0.01	N/S	0.31	N/S
TRJet >2.5 m/s	26 (16)	2 (12)	0.05	N/S	0.41	N/S
Sickle Cell Lung Disease*	21(13)	2 (12)	0.02	N/S	1.41	N/S
All respiratory complications	34(2)	4 (24)	0.13	N/S	0.38	N/S
<b>Cerebral complications</b>						
Stroke	16 (10)	4 (24)	2.89	N/S	3.07	N/S
Transient ischaemic attack	3 (2)	0 (0)	0.33	N/S	0.00	N/S
Silent infarcts	10 (6)	2 (12)	0.87	N/S	0.90	N/S
All cerebral complications	25 (3)	2 (29)	2.56	N/S	2.74	N/S
<b>Other complications</b>						
Leg ulcers	10 (10)	5 (29)	<b>5.21</b>	<b>0.02</b>	<b>4.68</b>	<b>0.03</b>
Avascular necrosis	25 (16)	5 (29)	1.19	N/S	0.01	N/S
End stage renal failure	5 (3)	1 (6)	0.38	N/S	0.03	N/S
At least 1 complication	76 (47)	13 (81)	<b>6.75</b>	<b>0.009</b>	2.91	N/S

\* Sickle cell lung disease diagnosed on CT findings

\$ Corrected for age, sex and alpha thalassaemia genotype

N/S = non-significant SCA = Hb SS and Hb S $\beta^0$  patients



## 5.5 Discussion

We present data on a well characterised cohort of patients with sickle cell disease and known Duffy phenotype, derived from genotyping for the promoter polymorphism. Our results confirm the findings of previous groups that Duffy negative patients have lower WBCs and neutrophil counts. Duffy negative and Duffy positive patients appear to be able to mount an increase in WBC during acute events. There is no difference in the amplitude of the WBC or neutrophil count from steady-state to acute state dependant on Duffy status, therefore it is unclear if Duffy status would have any effect on incidence or duration of vaso-occlusive episodes. However the time to readmission was significantly smaller in Duffy positive patients, which may be a reflection of their underlying higher WBC, with the role of WBC/RBC/vessel wall interactions known to play important role in vaso-occlusive episodes in SCD. Further investigation on the effect of the Duffy phenotype on blood flow in sickle cell patients could be explored.

Unlike Afenyi-Annan et al, we found that the degree of micro-albuminuria was not significantly associated with Duffy status, although cystatin C (a marker of renal function) was significantly associated. This needs to be explored further, correcting for other known influences on renal function such as the role of haemolysis and other genetic polymorphisms (see Chapter 6). Presence of at least one organ affected by SCD appeared at first to be strongly linked to the Duffy positive phenotype but this disappeared in the multivariate analysis due to the influence of age. Prevalence of leg ulcers was significantly higher in the Duffy positive group and persisted after multivariate analysis. The incidence of leg ulcers in the Jamaican population is known to be higher than that seen in other groups of SCD patients studied (Clare, *et al* 2002, Serjeant 1974). Serjeant has postulated that this is multi-factorial with impaired venous drainage, direct infarction of the skin and social factors (low socio-economic status, incidence of insect bites) all potentially playing a role. The Duffy positive phenotype could influence this; speculatively, the group with the higher WBC and neutrophil count (i.e. Duffy positive) potentiating inflammation and predisposing to skin infarction.

There are limitations to our study. Our numbers are small for some complications (including leg ulcers) and our follow-up period for the admission cohort was limited to 2 years. It also does not include data on Accident and Emergency attendances or the number of painful episodes patients have at home. Admission to hospital and duration of stay are also well known to be influenced by a range of cultural and social issues. Despite this our data shows novel associations between Duffy phenotype and markers of disease severity which would benefit from further investigation.

In summary, there are no clear results using Duffy phenotype as a predictor of disease severity in isolation, but there are certainly indications from our data that it could be included in a panel of predictors. Further work is required to validate the hypothesis that the raised WBC associated with Duffy positive phenotype could potentiate increased rates of vaso-occlusion and potentially be a risk factor for the development of leg ulceration.

## **Chapter 6 Renal Impairment in Sickle Cell Disease**

### **6.1 Introduction**

Renal impairment is common in patients with sickle cell disease (SCD), the frequency increasing with age. As life expectancy continues to improve in SCD patients (Platt, *et al* 1994), we can expect the proportion of those suffering from chronic end organ damage, to increase (Serjeant, *et al* 2007). In a recent study of Jamaican patients, 44% of those aged between 40 and 60 years of age demonstrated a greater than 50% increase in their serum creatinine over time. In those aged 60 or older, 43% had chronic renal failure as the most common cause of death (Serjeant, *et al* 2009). This is a significant health burden but outcomes in other causes of chronic kidney disease (CKD) have been improved by treatment of the underlying precipitator and also general management of the chronic kidney disease (Ritz, *et al* 2010, Sarnak, *et al* 2005, Schrier, *et al* 2002). Population studies have shown that the cumulative lifetime risk of developing chronic renal disease is higher in the African-American population (7.5%) than Americans of European heritage (2.5%) (Kiberd and Clase 2002) leading to exploration of genetic polymorphisms associated with and potentially related to underlying mechanisms of renal disease. Knowledge of patterns of presentation and these predisposing genetic factors in African-American patients without SCD could be applied to those with SCD, as these patients are from similar ethnic backgrounds, enabling earlier interventions and therefore potentially better outcomes for patients.

#### **6.1.1 Pathogenesis of Sickle Cell Nephropathy**

There is no pathognomic presentation of sickle cell nephropathy (SCN) and indeed it shares histopathological features with other causes of proteinuric renal disorders which need to be excluded as contributing causes. Focal segmental glomerular sclerosis (FSGS) is the pattern most commonly found in SCD and is a clinical syndrome involving podocyte injury and glomerular scarring. Patient with SCD are just as likely to suffer from other causes of renal dysfunction, including diabetes, hypertension, hepatitis B and C, autoimmune disorders, myeloma and malignancy, and these should be actively looked for, and excluded, as part of patient screening.

As with other end-organ complications in SCD it is thought that both vaso-occlusion and haemolysis play a role in its pathogenesis. In the normal situation the medulla of the kidney is

maintained in a situation of relative hypoxia, a consequence of the poor blood flow that is required to maintain the solute gradient which is essential for the production of concentrated urine. Unfortunately this environment is fertile ground for the polymerisation of HbS and therefore sickling of the red blood cells (RBCs). This results in repeated vaso-occlusive events in the vasa recta (the vessels that supply the medulla of the kidney) and thus downstream ischaemia, tissue death and microvascular disease. The decrease in oxygen levels then leads to prostaglandin-induced vasodilation and an increase in renal plasma flow (Becton, *et al* 2010, Scheinman 2009). This results in one of the earliest manifestations of SCN, hyperfiltration, manifested by a glomerular filtration rate greater than 140 ml/min/1.73m<sup>2</sup>. With increasing patient age, this begins to fall and as described above, a proportion of patients will develop significant renal impairment. The glomerular filtration rate is the basis for the classification of CKD into its various sub-types (see Table 18).

Table 18: The Kidney Disease Outcomes Quality Initiative (KDOQI) stages of chronic kidney disease. The addition of suffix p indicates significant proteinuria (albumin:creatinine ratio  $\geq 30$  mg/mmol or protein:creatinine ratio  $\geq 50$ mg/mmol); suffix T indicates the patient has had a renal transplant; suffix D indicates the patient is on dialysis.

Stage of CKD	Glomerular filtration rate (ml/min/1.73m <sup>2</sup> )	Description
1	$\geq 90$	Normal kidney function but urine findings or structural abnormalities or genetic trait point to kidney disease
2	60 – 89	Mildly reduced kidney function, and other findings (as for stage 1) point to kidney disease
3a	45 – 59	Moderately reduced kidney function
3b	30 – 44	Moderately reduced kidney function
4	15 – 29	Severely reduced kidney function
5	< 15 or on dialysis	Very severe, or end stage kidney failure

In addition to vaso-occlusion, chronic haemolysis is the other fundamental pathological process that occurs in SCA. In children with SCA, significant associations have been found between proteinuria with low haemoglobin (Hb) and high lactate dehydrogenase (LDH) levels, both markers of haemolysis (Becton, *et al* 2010, Gurkan, *et al* 2010). Haemolysis leads to a decrease in the availability of nitric oxide. Nitric oxide controls vasodilation via binding of soluble guanylate cyclase, leading to the conversion of GTP to cGMP, which in turn relaxes

vascular smooth muscle causing vasodilatation. Plasma Hb released during intravascular haemolysis leads to NO consumption, thus in SCD a state of reduced endothelial NO bioavailability exists causing impairment of downstream homeostatic vascular functions of NO resulting in vasoconstriction, and endothelial proliferation. Again, the relative ischaemic environment of the kidney makes it vulnerable to the results of NO depletion. There is clinical evidence of an association between the degree of haemolysis and renal impairment in both children and adults (Becton, *et al* 2010, Day, *et al* 2012, Gurkan, *et al* 2010).

Eventually, progressive nephropathy results in a failure of the “sieve” of the nephron leading to proteins leaking into the urine. There are recognised thresholds for the levels of proteinuria which have been correlated in diabetic renal disease to the need for intervention to preserve renal function. For example, microalbuminuria (albumin:creatinine ratio [ACR] of  $\geq 4.5$ ) is an early manifestation of SCN. In other nephropathies, this is known to correlate with an increase in all-cause mortality (Chronic Kidney Disease Prognosis, *et al* 2010). Eventually the level of protein in the urine increases and becomes frank proteinuria (protein:creatinine ratio [PCR]  $\geq 50$ ). Formal measurements of ACR are not available in all centres and therefore urine dipsticks are used instead. A positive urine dipstick (1+) correlates approximately to an ACR of 30 (see Table 19 for approximate correlations). Measurements of ACR/PCR are not reliable during an acute pan episode or menstruation, in steady state the results are more reproducible than 24 hour urine protein collections (due to improved reliability of sample collection, 24 hour collections often being underfilled).

Table 19: Various methods exist of measuring renal protein loss. This table shows the equivalent results between urine dipstix result, albumin:creatinine ratios and daily mass of proteinuria, as measured by 24 hour urine collection

<b>Urine dipstix result</b>	<b>Albumin:creatinine ratio (mg/mmol)</b>	<b>Daily mass of proteinuria (g/day)</b>
1+	30	<0.5
2+	100	0.5 - 1
3+	300	1 – 2
4+	2000	> 2

### 6.1.2 Treatment Options in Sickle Cell Nephropathy

As with the treatment of other end-organ complications in SCD there are 2 treatment options, a) simple transfusion or red cell exchange or b) hydroxycarbamide therapy. The evidence for

benefit of the use of chronic transfusion therapy in protecting patients from SCN is very sparse, with Alvarez *et al* showing some protective benefit when transfusions were commenced prior to the age of 9 years (Alvarez, *et al* 2006). This finding has not been replicated with Becton *et al* showing no effect on the proportion of children developing microalbuminuria (Becton, *et al* 2010). The data for the use of hydroxycarbamide is equally disappointing with the recently published BABY HUG study showing that hydroxycarbamide was equivalent to placebo in preventing the progression and development of hyperfiltration in children aged 9 to 17 months (Wang, *et al* 2011) although this was probably due to the study period being too short.

An important aspect of treatment for all forms of CKD is control of hypertension. Control of hypertension with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor type II blockers has also been shown to reduce albuminuria and proteinuria and slow progress to renal failure in diabetic and non-diabetic patients (Ritz, *et al* 2010, Sarnak, *et al* 2005, Schrier, *et al* 2002). The experience in SCD is limited to small short term-studies (Falk, *et al* 1992, McKie, *et al* 2007), but the results have been encouraging in that proteinuria and hyperfiltration have both shown improvements. Thus, using the combination of these data, and applying the positive results from other causes of CKD, the focus of treatment of SCN involves the use of ACE inhibitors or angiotensin receptor type II blockers as intervention in cases of significant proteinuria. Our local guidelines recommend the introduction of these drugs when the patient has a urinary protein/creatinine ratio above 100mg/mmol. Consistent hypertension (systolic blood pressure of >140mmHg and diastolic blood pressure of > 90mmHg) should also be aggressively controlled. In advanced CKD secondary to SCN, haemodialysis may be required. Unfortunately in SCD this has a very poor prognosis, Powars *et al* (1991) reported a mean time to death of 4 years after being commenced on haemodialysis (Powars, *et al* 1991). Abbott *et al* in 2002, in an analysis of the US Renal Data System, showed that SCD was an independent risk factor for death (Abbott, *et al* 2002). Renal transplant appears to be a more successful method (from a mortality perspective) of renal replacement therapy than haemodialysis with significantly improved outcomes in the Abbott cohort analysis following transplantation. Encouragingly the 1 year rejection rate and graft survival were comparable to ethnically matched controls but there was a reduction in the 3 year graft survival rate in the SCD group, possibly due to sickling in the graft. Renal grafts are in short supply for all causes of end stage renal disease (ESRD) and therefore any strategies that can provide early prediction (and

therefore intervention) for those at risk of renal failure and reduce the need for all forms of renal replacement therapy would be beneficial.

### **6.1.3 Genetic Predictors**

Understanding genetic predictors for the development of renal dysfunction could enable the tailoring of monitoring programmes on a patient by patient basis. As with other complications of SCD, renal dysfunction is more common in those with Hb SS or HbS $\beta^0$  genotypes compared with those patients with Hb SC or Hb S $\beta^+$  genotypes. It is also more common in those patients who have a lower HbF level and those who have not co-inherited alpha-thalassaemia trait (see 0). As discussed in 1.4.4.7, SNPs in genes belonging to the *TGFB/BMP* pathway have been associated with sickle nephropathy (Nolan, *et al* 2007). For this project I focused on 3 genes and their polymorphisms. These genes have been chosen in the context of this study for different reasons and include: 1) *APOL1*. SNPs in the *APOL1* gene have been associated with the risk of developing chronic kidney disease in the non-sickle African American population, 2) *DARC*. The Duffy positive phenotype was shown to be associated with macroalbuminuria and other markers of renal dysfunction in a smaller study of our patients; 3) *HMOX1*. Polymorphisms in the promoter of *HMOX1* modulate the level of heme oxygenase that is involved in the metabolism of heme generated from haemolysis. Haemolysis has been associated with the development of microalbuminuria.

#### **6.1.3.1 Apolipoprotein 1 (APOL1)**

Recent work by various groups has shown strong associations between SNPs in the *APOL1* gene and the risk of developing chronic kidney disease secondary to human immunodeficiency virus (HIV) associated nephropathy, idiopathic FSGS and hypertensive renal failure in the African American population (Genovese, *et al* 2010a, Kao, *et al* 2008, Kopp, *et al* 2008, Tzur, *et al* 2010). There are 2 alleles of interest in the *APOL1* gene, G1 and G2 (Genovese, *et al* 2010a) (see Table 20).

Table 20: Location and frequency of the APOL1 SNPs in focal segmental glomerular sclerosis (FSGS) and Yoruba populations. Data is shown from Genovese *et al* regarding increased SNP frequency in both cases of FSGS and ESRF of all causes. Also shown is the odds ratio (OR) or the strength of the association of number of inherited risk alleles with renal disease. NB: The risk allele is also the minor allele i.e. rs 73885319 (G) and rs71785313 (del)

	G1	G2
<b>Location</b>	Chr22 last exon of APOL1	Chr22 last exon of APOL1
<b>SNP rs number</b>	rs73885319 (S <sup>342</sup> →G <sup>342</sup> ) in LD with rs60910145 (I <sup>354</sup> →M <sup>354</sup> )	rs71785313 (6bp deletion – N <sup>388</sup> and Y <sup>389</sup> )
<b>Yoruba data</b>	38%	8%
<b>Genovese <i>et al</i> (Genovese, <i>et al</i> 2010b)</b>		
FSGS		
Cases n = 192	52%	23%
Controls n = 176	18%	15%
p value	1.07x10 <sup>-23</sup>	4.38x10 <sup>-7</sup>
End stage renal failure all causes		
Cases n = 1002	41%	21%
Controls n = 923	21%	13%
p value	1.1x10 <sup>-39</sup>	8.8x10 <sup>-18</sup>
OR 1 allele	1.04-1.26	1.04-1.26
OR 2 alleles vs. none	7.3-10.5	7.3-10.5
OR 2 alleles vs. 1	5.8 (4.5-7.5)	5.8 (4.5-7.5)

These areas containing either allele are only separated by 140 base pairs (see Figure 15) and studies have shown that patients inherit either *APOL1* G1 or G2 minor alleles on a chromosome, but not both. Genovese *et al* have shown that the inheritance of 1 risk allele, A→G in G1 or the 6 base pair deletion in G2, is associated with only a small increase in renal disease risk (OR = 1.26) compared to the inheritance of 2 risk alleles (either homozygous for the G1 or G2 SNPs or compound heterozygous) which led to a significantly increase risk of renal disease (OR 7.3) compared to wild-type.

ApoL1 is an apoprotein component of high density lipoprotein found in human serum, and is synthesised mainly in the liver, pancreas, kidney and brain. Part of its function is involved in the lysis of *Trypanosoma brucei brucei* and it is encoded by the *APOL1* gene found on chromosome 22. *Trypanosoma* is a parasitic organism found in west, southern and eastern Africa and is transmitted from animal to human hosts by insects, classically the tsetse fly. It causes a potentially fatal syndrome known as “sleeping sickness” in humans. Interestingly plasma from patients with the *APOL1* variants retains activity against *Trypanosoma* species that



have adapted to evade the effects of wild-type ApoL1. Work by Genovese *et al* has shown *in vitro* that serum from patients with at least 1 risk allele had active lytic properties against these previously resistant species. This provides a convincing argument as to an infective drive for these genes to be perpetuated in the population, despite their potential negative effects on renal function. Currently there is no knowledge of the mechanism by which the *APOL1* variants contribute to the pathogenesis of kidney disease and this is the subject of much on-going research worldwide.

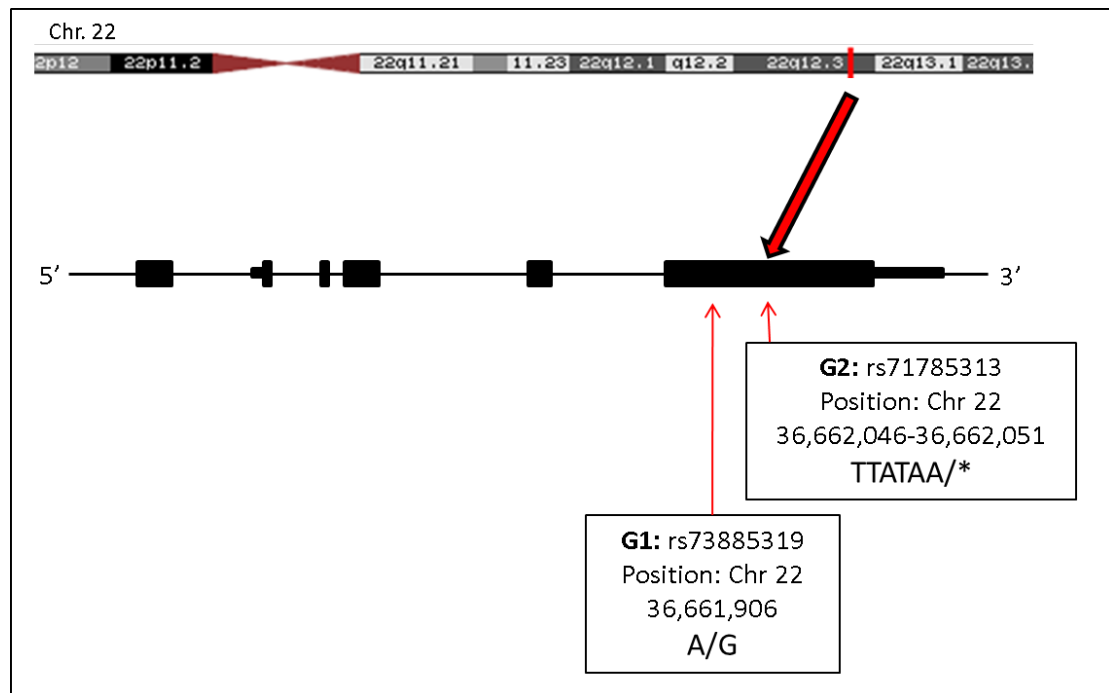


Figure 15: Structure of the *APOL1* locus indicating the G1 and G2 SNPs associated with increased risk of renal dysfunction.

Ashley-Koch *et al* recently published data from an American cohort of patients with SCD, showing that the risk alleles were significantly associated with proteinuria. However this group did not have longitudinal measures of renal function and used protein dip-stick only as a measure of albuminuria ( $ACR \geq 30$ ), as opposed to the more sensitive albumin:creatinine ratio used at King's College Hospital. No statistically significant associations were seen with GFR (Ashley-Koch, *et al* 2011). Haemolytic parameters, which have been shown to be associated with the development of albuminuria were also not included in the analysis (Becton, *et al* 2010, Day, *et al* 2012, Gurkan, *et al* 2010, Maier-Redelsperger, *et al* 2010).

### 6.1.3.2 The Duffy Antigen Receptor for Chemokines

The structure and function of the Duffy Antigen Receptor for Chemokines (DARC) is discussed in detail in Chapter 5. We have shown that the Duffy positive phenotype, a sign of European admixture and associated with a raised white cell count, is associated with the development of macroalbuminuria and other markers of renal dysfunction including a raised cystatin C.

### 6.1.3.3 Heme oxygenase

Work from our own group has recently shown a strong association between the severity of haemolysis and degree and prevalence of microalbuminuria (Day, *et al* 2012). Heme oxygenase is a rate-limiting enzyme in heme degradation, leading to the generation of free iron, biliverdin, and carbon monoxide. Induction of heme oxygenase-1 (*HMOX-1*) in SCD is implicated in the antioxidant defence mechanism and can modulate vascular function (Bains, *et al* 2010, Belcher, *et al* 2010, Siciliano, *et al* 2011). Reduced levels of heme oxygenase may result in reduced nitric oxide availability, endothelial dysfunction and vasculopathy. Transcriptional activity of the gene appears to be effected by a polymorphism in the promoter region consisting of multiple GT repeats (GT<sub>22-42</sub>) with peaks at 23 and 30 repeats (although some groups describe a tri-modal distribution), reduced expression being associated with a larger number of repeats. Cell lines containing fewer repeats appear to have increased transcriptional activity, possibly due to conformational change, and have greater oxidative stress induced *HMOX-1* gene expression and higher HO-1 enzyme activity (Hirai, *et al* 2003). Bean *et al* investigating the effect of *HMOX-1* promoter repeats on causes of admissions in a paediatric population found that children with shorter alleles ( $\leq 25$  repeats) had lower rates of hospitalisation with acute chest syndrome. However no relationship was identified between allele length and admission with pain (Bean, *et al* 2012). In this study we postulate that variability in heme oxygenase levels and the *HMOX-1* gene promoter could be associated with SCN.

## 6.2 Renal disease and Haemolysis in sickle cell disease

As previously discussed in 1.3.1.5, studies have questioned whether renal dysfunction in sickle cell disease (SCD) is linked to haemolysis-associated vasculopathy. Clarification of this could lead to identification of an “at risk” population. We investigated markers of haemolysis and markers of renal function and endothelial damage in the SCD population at King’s College Hospital.

### 6.2.1 Objectives

- a) To characterise patterns of endothelial damage and renal function in the SCD population at King’s College Hospital
- b) To look for relationships between haemolytic markers and markers of renal function (estimated glomerular filtration rate, eGFR) and endothelial damage (albumin creatinine ratio, ACR).

These data has been published. See appendix. (Day, *et al* 2012)

### 6.2.2 Results

Data pertaining to renal dysfunction (microalbuminuria and eGFR) were collected on 424 patients (253 HbSS, 7 HbS $\beta^0$  thalassaemia, and 164 HbSC) of which 255 (60%) were female. Patients with HbSS and HbS $\beta^0$  thalassaemia (HbS $\beta^0$ ) were analysed as one group but patients with HbSC were analysed separately. 37% of patients with Hb SS/S $\beta^0$  had microalbuminuria (as defined by an ACR  $\geq 4.5$  mg/mmol). In patients with Hb SS/S $\beta^0$  significant positive correlations for degree and prevalence of albuminuria were found with age, reticulocyte count, LDH and bilirubin. Degree and prevalence of albuminuria correlated negatively with Hb level. The presence of co-existent alpha thalassaemia appeared protective for the degree and prevalence of albuminuria. 71% of the HbSS/HbS $\beta^0$  group had at least one eGFR of  $\geq 140$  ml/min/1.73m<sup>2</sup> during the study period. eGFR was found to be significantly negatively correlated with age, reticulocyte count and bilirubin.

26% of the HbSC group had at least one sample with an ACR  $\geq 4.5$  mg/mmol (i.e. microalbuminuria). There was no significant association between degree of albuminuria and age, although there was an increase in the prevalence of microalbuminuria in this group with age. There was no significant association between prevalence or degree of microalbuminuria and other haemolytic variables. The presence of alpha thalassaemia trait had no influence on the development of albuminuria in the HbSC population. 24% had at least one eGFR of  $\geq 140$

ml/min/1.73m<sup>2</sup> during the study period. Estimated GFR was significantly associated with reticulocyte count although less strongly than in the HbSS/HbSβ<sup>0</sup> group.

Table 21: Table 21a: Relationship of haemolytic parameters to ACR in HbSS + Sβ<sup>0</sup> group.  
Table 21b: Relationship of haemolytic parameters to MDRD eGFR in HbSS + HbSβ<sup>0</sup> group

a)	UNCORRECTED FOR COVARIATES					CORRECTED FOR COVARIATES	
	Haemolytic parameters	SD	Degree of albuminuria		Microalbuminuria	Degree of albuminuria	
			% change per SD (95%CI)	p-value		% change per SD (95%CI)	p-value
Reticos (x109/L)		135.3	10.5 (1.1, 20.8)	0.02	1.3 (0.98, 1.77)	6.2 (-3.9, 17.3)	0.2
Hb (g/dL)		1.4	-18.6 (-27.8, -8.3)	0.0005	0.5 (0.31, 0.65)	-19.6 (-29.6, -8.1)	0.001
LDH (IU/L)		162.5	47.3 (32.1, 64.2)	<0.00001	2.6 (1.81, 3.79)	43.6 (27.5, 61.7)	<0.00001
Bilirubin (μmol/L)		0.6	60.8 (34.6, 92.2)	<0.00001	2.2 (1.22, 3.84)	55.9 (29.2, 88.0)	<0.00001
RBC-Hb/RET-Hb		0.5	-27.2 (-43.4, -6.2)	0.01	0.08 (0.03, 0.20)	-27.3 (-46.0, -2.2)	0.03
RBC-Hb		0.2	-44.6 (-57.3, -28.1)	<0.00001	0.04 (0.01, 0.19)	-49.7 (-62.2, -32.1)	<0.00001

b)	UNCORRECTED FOR COVARIATES				CORRECTED FOR COVARIATES	
	Haemolytic parameters	SD	Absolute change per SD (95%CI)		p-value	p-value
			Absolute change per SD (95%CI)	p-value		
Reticos (x109/L)		135.3	5.8 (3.4, 8.1)	<0.00001	8.3 (5.8, 10.8)	<0.00001
Hb (g/dL)		1.4	1.6 (-0.3, -0.1)	0.3	1.1 (-2.1, 4.4)	0.5
LDH (IU/L)		162.5	-2.7 (-5.6, 0.2)	0.2	-2.3 (-5.3, 0.6)	0.1
Bilirubin (μmol/L)		0.6	3.0 (-1.6, 7.5)	0.2	5.9 (1.2, 10.5)	0.01
RBC-Hb/RET-Hb		0.5	-12.3 (-20.5, -4.0)	0.003	-8.1 (-12.848, 9.062)	0.1
RBC-Hb		25.9	-1.1 (-10.5, 8.4)	0.8	0.18 (-12.848, 9.062)	0.7

### 6.2.3 Discussion

Sickle cell nephropathy (SCN) is an important cause of morbidity and mortality in patients with SCD (Powars, *et al* 1991), characterised by hyperfiltration at a young age, followed by a gradual reduction in GFR and worsening proteinuria. Using a broad definition of microalbuminuria and hyperfiltration, SCN is common amongst a UK sickle population, with a combined incidence of hyperfiltration (as defined by an eGFR of  $\geq 140$  ml/min/1.73 m<sup>2</sup>) of 71%, and of

microalbuminuria (ACR of  $\geq 4.5$  mg/mmol) of 37%, making SCN one of the most common complications of SCA. The data also demonstrates associations between both degree and prevalence of albuminuria and all markers of increased haemolysis.

Given the reduction in haemolysis, it is not surprising that co-inheritance of alpha thalassaemia has a protective effect against albuminuria in SCA patients, with a negative association between number of deleted alpha-genes and the degree of albuminuria and prevalence of microalbuminuria, replicating previous findings. Clinically, raised markers of haemolysis may prove to be useful in identifying those at increased risk of developing sickle cell nephropathy.

### 6.3 Genetic predictors of renal dysfunction in Sickle Cell Disease

As previously discussed in 6.1.3, various polymorphisms have been implicated in the development of renal dysfunction in SCD. These findings, if positive could add to the development of a panel of genes to screen for disease severity. I investigated the associations between 3 candidate genes of interest and the development of SCN.

#### 6.3.1 Objective

- a) **To examine associations between genetic polymorphisms of the *APOL1* gene, the *DARC* gene and *HMOX1* gene with the development of renal dysfunction within the SCD cohort at King's College and St Thomas' and Guy's Hospital NHS trusts**

#### 6.3.2 Methods

DNA was extracted from buffy coats obtained via the sickle cell gene bank (REC 07/H0606/165) and prepared using standard methods.

##### 6.3.2.1 Genotyping of *DARC* promoter polymorphism

Presence or absence of this polymorphism was ascertained using a Taq Man allelic discrimination assay from ABI biosystems. Individuals with genotypes T/T or C/T were assumed to have Duffy positive phenotypes whereas those with genotype C/C were assumed to be Duffy negative.

##### 6.3.2.2 Genotyping of the *APOL1* G1 and G2 polymorphisms

Primers were designed using sequences exported from the UCSC genome browser. Areas with multiple repeats and known polymorphisms were avoided as potential sites of primer annealing. The following criteria were used for primer selection: 70 base pair product length, false priming at less than 150 points and a G+C: A+T ratio of close to 50%. Specificity was checked using the Primer-BLAST tool on the National Centre for Biotechnology Information (NCBI) website. The resulting custom designed primers and probes for TaqMan real time PCR analysis were ordered from Applied Biosystems (UK).

##### **G1: rs73885319**

The final TaqMan assay consisted of:

Forward primer: 5'-GAAATGAGCAGAGGAGTCAAGCT-3'

Reverse primer: 5'-AGGTAGACTACATCCAGCACAAGA-3'

Probe with reporter VIC: 5'-CCCCTGTAAGCTTCTT-3' (A – wild type)

Probe with reporter FAM 5'-CCCTGTAGGGCTTCTT-3'. (G – risk allele)

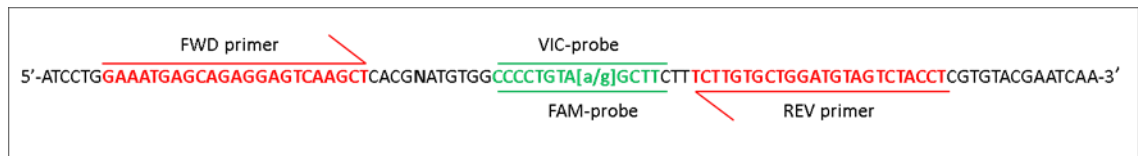


Figure 16: Primers and probes for *APOL1* G1 Taqman allelic discrimination assay

## G2: rs71785313

The final TaqMan assay consisted of:

Forward primer sequence: 5'-GCTCAGGAGCTGGAGGAGAA-3'

Reverse primer sequence: 5'-CCTGCCCTGTGGTCACA-3'

Probe with reporter VIC: 5'-CCTGCAGAATCTTATAATT-3' (TAATT – wild type)

Probe with reporter FAM: 5'-CCTGCAGAATCTTATTG-3' (del TTATAA – risk allele)



Figure 17: Primers and probes for *APOL1* G2 Taqman allelic discrimination assay

Genomic DNA extracted from peripheral blood leukocytes, was diluted to a 10ng/μl concentration. 1 μl (10 ng) per reaction of DNA was placed into 384 well plates centrifuged at 3000 rpm and dried at room temperature for 24 hours. Universal TaqMan Master Mix (Applied Biosystems, UK), was used with primers and probes as recommended by the manufacturer to a final reaction volume of 3μl. Real-time quantitative PCR analysis was performed using Applied Biosystems (ABI) PRISM® 7900HT Sequence Detection System to determine presence or absence of the G1 risk polymorphism (G) and the G2 risk allele (del TTATAA).

The results for the G2 6 base pair deletion using Taqman genotyping showed a somewhat unusual genotype clustering (Figure 4), i.e., drawn-out clouds that were located in the centre of the plot, rather than near the axes. Still, the three genotype clusters are clearly distinct and allele calling could be performed with confidence.

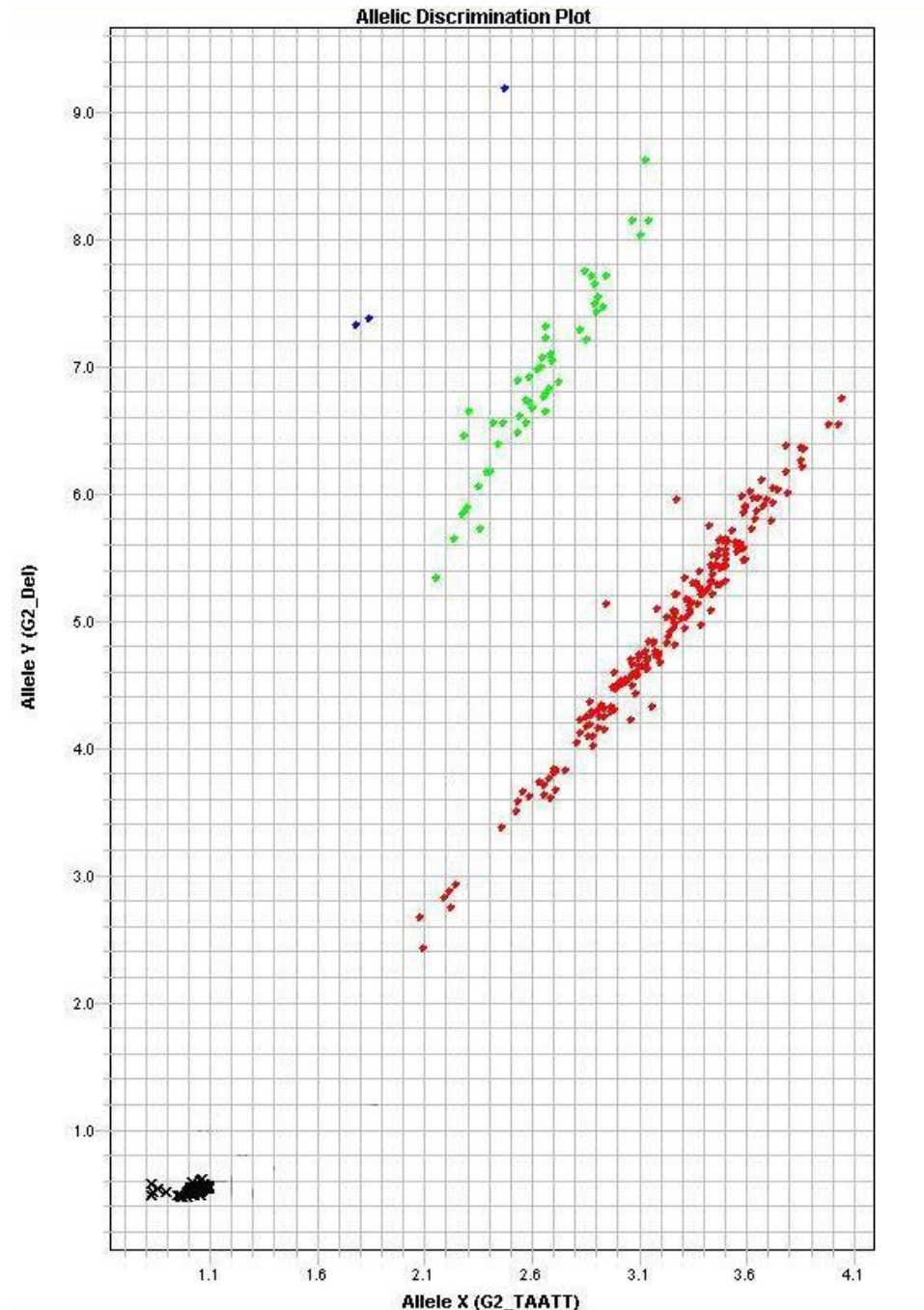


Figure 18: Allelic discrimination plot for *APOL1* G2 Taqman assay.

Dark blue dots indicate homozygous inheritance of the 5bp (TAATT) deletion. Red dots indicate homozygous inheritance of the wild type genotype (no deletion TAATT). Green dots indicate heterozygous patients.



#### 6.3.2.2.1.1 PCR of *APOL1* G2 allele

The results were confirmed by Sanger sequencing in 10 selected patients with a mixture of genotypes for the *APOL1* G2 allele. PCR of the region of interest was performed and the products sequenced using capillary electrophoresis on the ABI 3100 sequencer using a Big Dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, US). Custom designed primers for PCR and sequencing were ordered from Applied Biosystems (UK).

Primer sequences were:

Forward: 5' – CATGCCTCAGCCTCAGCCC – 3'

Reverse: 5' – CCTGGCCCCTGCCAGGCATA – 3'

50ng of genomic DNA extracted was placed into a 96 well plate, centrifuged and dried at room temperature for 24 hours. 25 µl reactions were set up with 800pmol of the forward primer, 8000pmol of the reverse primer, 0.2 mM of dNTPs, 0.5U TaqGold (Applied Biosystems, UK), 2.5 mM of MgCl<sub>2</sub> and 2.5µl of PCR buffer (Applied Biosystems, UK). Cycling conditions were adapted from the standard ABI PCR protocol and are shown in Table 22.

Table 22: Cycling conditions for PCR amplification of *APOL1* G2 allele using Tetrad PCR machine with heated lid

Temperature (°C)	Time	
95	12 mins	
94	30 secs	9 cycles
65	30 secs	
72	30 secs	
89	30 secs	
65	30 secs	19 cycles
71	30 secs	
72	10 mins	

#### 6.3.2.2.1.2 PCR product purification

The resulting PCR products were stored at +4°C in a sealed 96 well plate. The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega), a membrane based method.

#### 6.3.2.2.2 Cycle sequencing

Master mix was made as below for forward and reverse primers (using the same primers as used for the PCR) in separate reactions i.e. 2 reactions required per sample. 20 µl reactions were set up with 100ng of the template, 3 pmol of the forward and reverse primers, 1 µl Big Dye terminator reaction mix and 4 µl buffer. Thermocycle was performed on Tetrad with heated lid. The cycling protocol used was as follows: 15 sec, 96 °C; 25 cycles of 10 sec, 96 °C, ramping 1 °C/sec to 50 °C, 5 sec, 50 °C, ramping 1 °C/sec to 60 °C, 3:30 min, 60 °C; finally cooling to 4 °C.

Sequencing products were transferred into 1.5 ml microcentrifuge tube and ethanol precipitated: 80 µl ethanol mix was added (per tube: 16 µl de-ionized water + 64 µl 95% ethanol) to each tube and left at room temperature for 15 min. The microcentrifuge tubes were spun for 20 min at 28000 x g at 4 °C. The supernatant was removed and the precipitate washed with 250 µl of 70% ethanol. The microcentrifuge tubes were spun for 10 min at 28000 x g at 4 °C. The supernatant was removed and the tubes containing the sequencing reaction air-dried for 30 min. The sequencing products were re-suspended in 20 µl formamide and transferred into a 96-well plate, denatured for 2 min at 95°C on a thermal cycler followed by rapid cooling to 4 °C. Sequencing products were separated and detected through capillary electrophoresis on a Applied Biosystems 3130 automated sequencer (using 36 cm capillaries). Data was analysed using Sequencher v 4.6 software (Gene Codes Corporation, Ann Arbor, MI USA).

The results of the Taqman allelic discrimination were confirmed by the Sanger sequencing (see Figure 19) and therefore the Taqman “calls” were used in the analysis.

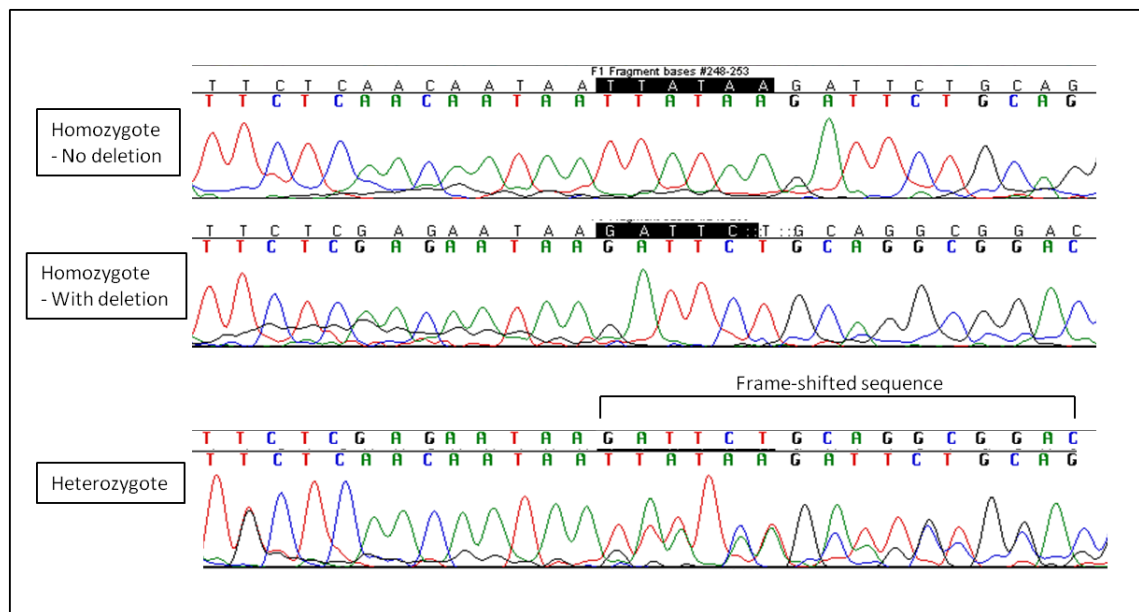


Figure 19: *APOL1* G2 genotypes were confirmed using Sanger sequencing.

The homozygote wild type (no deletion) shows the TTATAA sequence intact (see black highlighted section). The homozygote risk-allele shows the deletion with the subsequent sequence shifted to the left (see black highlighted section). The heterozygote shows a frame-shifted sequence from the deletion onwards as expected.

### 6.3.2.3 Microsatellite Genotyping of *HMOX1*

Microsatellites (also called short tandem repeats, STR) are repeating sequences of 2-6 base pairs of DNA. Microsatellites can affect gene function when they occur in functional sequence, such as promoters or other regulatory elements. The transcription of *HMOX-1* appears to be affected by a polymorphism in the promoter region consisting of a GT repeat sequence (GT<sub>22-42</sub>). (See Figure 20). As previously discussed (6.1.3.3), the greater the number of repeats in the promoter region, the lower the efficiency of transcription and therefore lower levels of heme oxygenase.

For these experiments higher resolution genotyping was performed using PCR with fluorescent primers and subsequent detection of fragment length using capillary electrophoresis on the ABI 3100 sequencer. The PCR products were labelled with FAM (a fluorescent dye), which was attached to the forward primer. These labelled DNA fragments were combined with an

appropriate size standard to migrate through polymer filled capillaries past a laser beam which excites them. The separation is based on the differences in electrophoretic mobility according to size. A charged coupled device (CCD) camera converts the fluorescence into digital data that is processed by the 3100 data collection software. Data analysis and subsequent allele sizing and scoring was performed using ABI Genemapper software. Using control samples of known size it was possible to calculate the product size of the unknown samples.

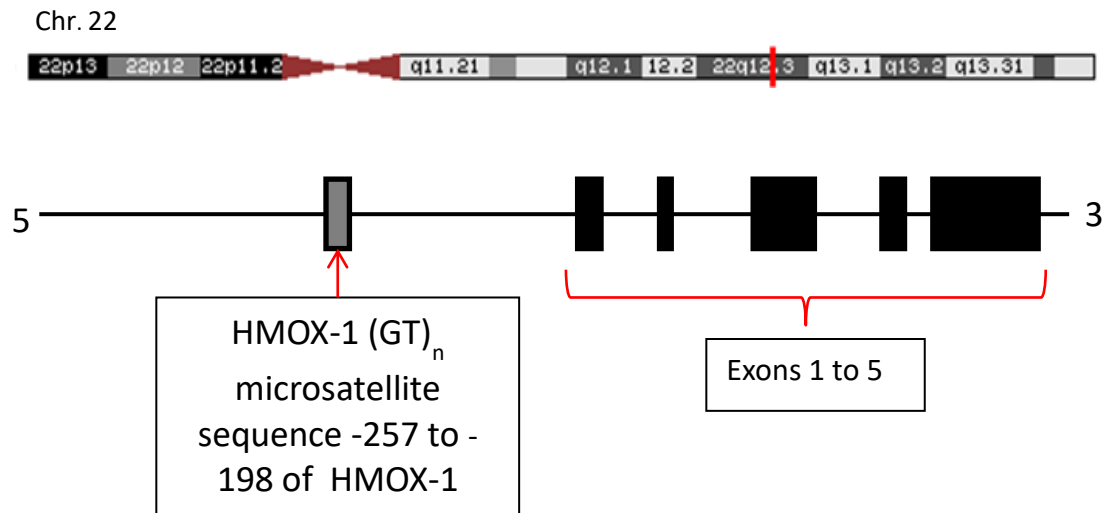


Figure 20: Structure of the *HMOX-1* locus indicating the (GT)<sub>n</sub> sequence located between base -257 and -198 of the *HMOX-1* promoter.

PCR was performed to amplify a fragment containing the microsatellite repeat, resulting in products of various lengths according to genotype. Primers were used as previously published (Vasavda, *et al* 2007), the sequences are shown below.

Forward – FAM\*5' AGAGCCTGCAGCTTCTCAGA 3'

Reverse – 5' ACAAAGTCTGGCCATAGGAC 3'

### 6.3.3 Data Management and Statistical Analysis

Biological data including haemoglobin (Hb), lactate dehydrogenase (LDH), WBC, neutrophil count, HbF, reticulocyte count, ferritin, creatinine, urine albumin creatinine ratio (ACR), cystatin C and erythropoietin levels were collected from routine blood results in steady state clinic during a 12 year period from January 1<sup>st</sup> 2001 to December 31<sup>st</sup> 2012. Estimated glomerular filtration

rates (eGFRs) were calculated using the 4-point Modification of Diet in Renal Disease (MDRD) formula. Patient age at the time of data capture was recorded. Clinical data regarding hydroxycarbamide and transfusion therapy was also recorded for each sample. Variables were log transformed where appropriate to obtain a normal distribution. Data were manipulated in Excel (Microsoft, Seattle) and SPSS (IBM, New York). *HMOX1* promoter region microsatellite copy number was analysed as a continuous variable. *APOL1* genotyping results were treated as categorical variables using the number of risk alleles. The *DARC* promoter polymorphism was used to derive Duffy phenotype which was then used as a binary variable in analyses. The regression analysis for biological variables was performed in Stata 11 (Stata Corp) by Dr Tony Fulford (London School of Hygiene & Tropical Medicine, London). This analysis pooled all data from each individual allowing for dependency between multiple observations on the same individual using random effects models fitted either using generalised least squares or, when the dependent variable was either binary (logistic regression) or censored (interval regression), by maximum likelihood. All analyses corrected for sickle and alpha globin genotype.

#### **6.3.3.1 Definitions of Renal Impairment**

As previously discussed (6.1.1) the degree of proteinuria can indicate differing needs for intervention, therefore proteinuria was always treated as a binary variable in the analyses. Associations with presence or absence of microalbuminuria ( $ACR < 4.5$  = absent and  $ACR \geq 4.5$  = present) and associations with presence or absence of macroalbuminuria ( $ACR < 30$  or  $ACR \geq 30$ ). The latter corresponds to approximately 1+ of protein on urine dipstick test which was the measure of nephropathy used in previously published data (Ashley-Koch, *et al* 2011). Estimated GFR was treated as a binary variable with renal impairment defined as an MDRD eGFR  $\leq 60$ , which corresponds to stage 3 (or more severe) CKD, or moderately decreased renal function. Cystatin C is an emerging marker of renal function, however its availability is limited from centre to centre and is not available for historical data sets, making longitudinal analysis difficult. It meets the criteria for an ideal GFR marker i.e. stable production rate, circulating levels are not affected by other pathological changes and is freely filtered by the glomerulus without tubular reabsorption or secretion (Dharnidharka, *et al* 2002). It has not been validated in adults with SCD, although results in a paediatric population (Unal, *et al* 2013) did show good

correlation with standard methods of assessing sickle nephropathy. In our laboratory an abnormal result is  $\geq 1.0$  mg/L.

### 6.3.4 Results

#### Study group and sub groups

The study cohort consisted of 410 patients. 236 (58%) of the group were female and 174 (42%) male. Ages ranged from 16 to 82 years (mean 33 years). The clinical characteristics of the study group are outlined in Table 23. The proportions of the genotypes and clinical characteristics were similar to those of our smaller initial study into the effect of Duffy phenotype on WBC and the development of end-organ complications. The analyses relating to WBC and renal complications associated with Duffy phenotype as described in Chapter 5 were repeated to see if increased numbers (410 vs. 272) of patients led to an alteration in significance particularly in view of the trend towards a significant association between Duffy positive phenotype and macroalbuminuria.

Table 23: Summary of demographic data for study group used to examine the association of renal dysfunction with the three candidate genes

	<b>Whole cohort</b>
<b>Number of patients</b>	410
<b>Male: Female (%)</b>	174:236 (42:58)
<b>Mean age (range)</b>	33 (16 to 81)
<b>Sickle genotypes</b>	
Hb SS (%)	271 (66)
Hb SC (%)	121 (29)
Hb S $\beta^+$ (%)	15 (4)
Hb S $\beta^0$ (%)	3 (1)
<b>Alpha genotypes</b>	300 (73)
$\alpha\alpha/\alpha\alpha$ (%)	185 (62)
$\alpha\alpha/\alpha-$ and $\alpha-/ \alpha-$ (%)	94 and 17 (31 and 6)
$\alpha\alpha/\alpha\alpha\alpha$ (%)	4 (1)

### Genotyping results

Laboratory data was available for the entire cohort. The *DARC* promoter polymorphism (*DARC* -33 C/C) underlying the erythroid silent (Duffy negative phenotype) was determined by TaqMan in 395 patients. The Duffy phenotype was predicted to be negative based on homozygous inheritance of the polymorphism (*DARC* -33C/C) in 356 (90%) of patients and positive based on the presence of 1 or 2 *DARC* -33 T alleles (i.e. *DARC* -33T/C and -33T/T) in 35 (9%) and 4 (1%) patients, respectively. The *APOL1* G1 (A/G and G/G) and G2 (TTA TAA/del and del/del) risk allele polymorphisms were determined by TaqMan in 395 patients. 181 (46%) of patients had 1 risk allele (either G1 or G2) and 91 (23%) had 2 risk alleles. The remainder (123, 31%) had no risk alleles. All variants passed the Hardy-Weinberg test. Genetic data is summarised in Table 24.

Table 24: Duffy genotypes and phenotypes and *APOL1* genotypes and risk allele scores for whole group (including Hb S $\beta^+$ ) and genotype sub-groups. There appears to be no difference in allele frequency between the genotypes.

	Whole group	Sickle Cell Anaemia (Hb SS and Hb S $\beta^0$ )	Hb SC
<b>Number of patients (%)</b>	410	274	121
<b>DARC genotypes</b>	395	266	115
C/C	356 (90)	244 (92)	101 (88)
C/T	35 (9)	20 (7)	12 (10)
T/T	4 (1)	2 (1)	2 (2)
<b>Duffy phenotypes</b>	395	274	115
Duffy negative	356 (90)	244 (92)	101 (88)
Duffy positive	39 (10)	22 (8)	14 (12)
<b>APOL1 genotypes</b>	395	274	115
G1 A/A	188 (48)	137 (50)	58 (50)
G1 A/G	155 (39)	102 (37)	45 (37)
G1 G/G	52 (13)	39 (14)	12 (10)
G2 TTATAA/TTATAA	296 (75)	205 (75)	90 (78)
G2 TTATAA/del	94 (24)	66 (24)	23 (20)
G2 del/del	5 (1)	3 (1)	2 (2)
<b>APOL1 risk alleles</b>			
1 risk allele	181 (46)	118 (43)	52 (44)
2 risk alleles	91 (23)	67 (25)	22 (20)

392 patients were genotyped for the *HMOX1* promoter polymorphism (*HMOX1* GT<sub>n</sub>) using capillary electrophoresis. The total number of copy repeats per allele ranged from 22 to 43 (mean 32) and were found to be arranged in a roughly trimodal distribution as previously reported by other groups (see Figure 21).

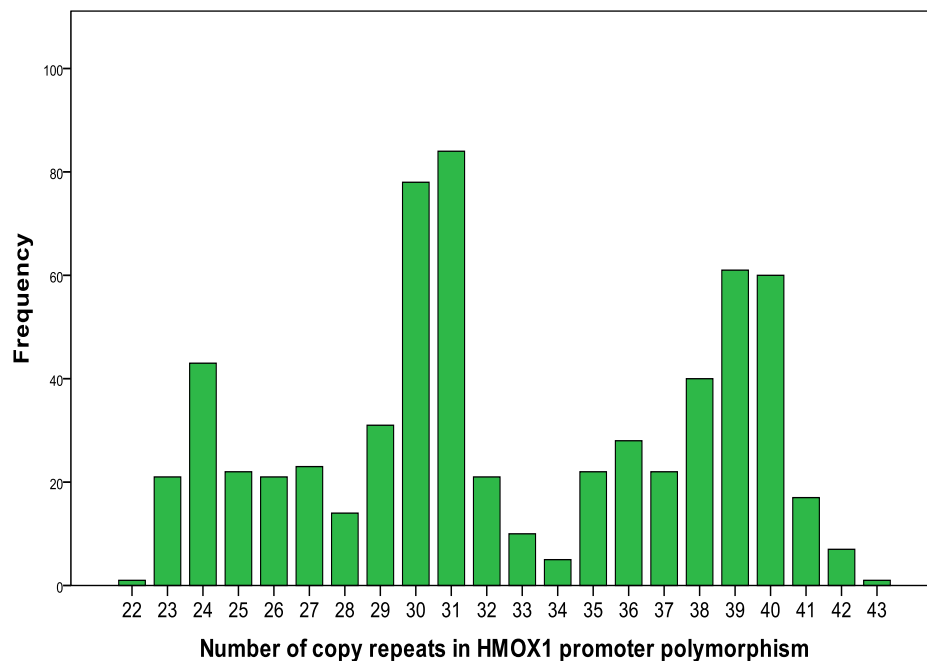


Figure 21: Histogram showing the distribution of single alleles in the *HMOX1* promoter polymorphism

### Statistical analysis

Logistic regression was performed by Dr Tony Fulford (London School of Hygiene and Tropical Medicine). This method (which was used for all analyses) was chosen to maximise the power of the dataset using multiple episodes from each patient instead of single data points or mean values. These analyses were corrected for age, gender, sickle and alpha genotype and whether the sample was taken when the patient was transfused or treated with hydroxycarbamide.

### Association of genotype with haemolytic variables and white blood count

As we have published, haemolysis appears to be a significant predictor of the development of microalbuminuria (see 6.2) (Day, *et al* 2012). The effect of sickle genotype and alpha-globin genotype on haemolysis is well documented, patients with SCA and those without alpha-trait



having a more haemolytic phenotype, with higher LDH, reticulocyte count and bilirubin and a lower haemoglobin (and being at a higher risk of developing proteinuria). Other polymorphisms could also have similar effects and we therefore wished to exclude any confounding from effects of our target genes on haemolysis, and therefore potentially on the development of renal impairment. We therefore examined our target genes to look for associations with LDH, bilirubin and reticulocyte count. A higher WBC is known to be associated with increased frequency of vaso-occlusion and therefore we also examined our target genes for associations with this parameter and to confirm our association with WBC and the Duffy positive genotype in a larger cohort (410 vs. 272 participants) than that performed in Chapter 5. Biological variables were available on the entire study group.

The results are summarised in Table 25. Unsurprisingly sickle genotype (effect for SCA) had the most significant association with all the parameters examined, with all the haemolytic variables increased in the presence of the SCA genotype. WBC was also increased. Presence of alpha trait also has a protective association with significant reduction in LDH and bilirubin. No significant effect was seen on reticulocyte count or WBC. *APOL1* had a negative association on LDH but not clinically significant. No other variables were significantly associated. In contrast increased number of *HMOX1* promoter repeats was associated with a higher LDH, although only to a clinically insignificant degree. A negative association was seen with WBC, each increase in repeat numbers causing a decrease in WBC by  $0.05 \times 10^9$ . All other associations were non-significant. The sole significant association with the presence of Duffy positive genotype was increased WBC (previously discussed Chapter 5). The significance is lower than that in our initial study, although the size of the effect is similar between the 2 groups.

Table 25: Summary of genotype influence on haemolytic variables and WBC. The effect refers to the co-efficient of the regression for each variable. Where log transformation was performed prior to analysis this was reversed to give a relevant effect in the standard units of the variable. Negative effect is indicated by a – sign.

Biological variable	Sickle genotype (effect of SCA)		Alpha genotype (presence of trait)		APOL1 (number of risk alleles)		HMOX1 (total number repeats)		DARC (positive phenotype)	
	Effect	p value	Effect	p value	Effect	p value	Effect	p value	Effect	p value
LDH	81.1	<0.0001	-11.8	0.003	-2.9	0.004	1.0	<0.0001		N/S
Bilirubin	85.9	<0.0001	-15.3	<0.0001		N/S		N/S		N/S
Absolute Retic	132.6	<0.0001		N/S		N/S		N/S		N/S
WBC	2.5	<0.0001		N/S		N/S	-0.05	0.01	1.2	0.02

### Association of genetic polymorphisms with development of microalbuminuria (ACR $\geq 4.5$ )

As previously published microalbuminuria was more common in patients with SCA compared to Hb SC (30% vs. 13% having at least 1 ACR  $\geq 4.5$ ) (Day, *et al* 2012) and co-inheritance of alpha thalassaemia trait is associated with a reduced risk of developing microalbuminuria (see Table 26). The additional influence of haemolytic variables and WBC over and above that of the presence of SCA appears to be minimal although statistically significant. However the presence of higher haemolytic markers (for example due to alpha-trait being co-inherited with SCA) appears to reduce the reno-protective effect of alpha-trait. None of the other polymorphisms were associated with the development of microalbuminuria.

Table 26: Summary of the influence of genotype taking into account the effects of haemolytic variables on the presence or absence of microalbuminuria (ACR  $\geq 4.5$ ) as the binary outcome variable.

Haemolytic variable used in analysis	Sickle genotype (effect of SCA)		Alpha genotype (presence of trait)		APOL1 (number of risk alleles)		HMOX1 (total number repeats)		DARC (positive phenotype)	
	Effect	p value	Effect	p value	Effect	p value	Effect	p value	Effect	p value
No haemolytic variables	2.45	<0.0001	-3.02	0.002		N/S		N/S		N/S
LDH	2.24	<0.0001	-0.65	0.01		N/S		N/S		N/S
Bilirubin	2.14	<0.0001	-0.62	0.02		N/S		N/S		N/S
Absolute Retic	2.44	<0.0001	-0.68	0.007		N/S		N/S		N/S

## Association of genetic polymorphisms with development of macroalbuminuria

Macroalbuminuria was also found frequently in our population. 6% of patients with Hb SC and 14% of patients with SCA having ACRs  $\geq 30$ . As discussed above Ashley-Koch *et al* have shown an association between the presence of two *APOL1* risk alleles and macroalbuminuria in an African-American cohort. We therefore repeated this analysis, while taking into account the association with haemolytic variables and other factors known to influence the development of a raised ACR (sickle genotype, age) as well as the other genetic areas of interest.

Duffy phenotype was significantly associated with the development of macroalbuminuria; coefficient 2.25  $p = 0.006$  (95% CI 0.64 – 3.87). This association persisted when additional correction occurred for the individual haemolytic variables and WBC. This appears to be an effect independent of haemolysis or WBC. Indeed, presence of the Duffy positive phenotype appears to be as significant an association as SCA in the development of macroalbuminuria in this larger cohort (our original smaller cohort only showed a trend towards significance with a  $p$  value of 0.06 see Chapter 5). Interestingly the addition to the analysis of bilirubin appeared to enhance the influence of Duffy on the development of macroalbuminuria and increase its significance, perhaps indicating at the dual haemolytic and vaso-occlusive pathology behind the development of this complication. No significant association was found with *APOL1* risk alleles or *HMOX1* promoter polymorphism (data summarised in Table 27). This is contrast to the data from Ashley-Koch *et al*, however our analysis corrected for haemolytic variables and WBC which have both been previously shown to influence the development of renal dysfunction.

Table 27: Summary of the influence of genotype taking into account the effects of haemolytic variables on the presence or absence of macroalbuminuria (ACR  $\geq 30$ ) as the binary outcome variable.

Biological variable used in analysis	Sickle genotype (effect of SCA)		Alpha genotype (presence of trait)		APOL1 (number of risk alleles)		HMOX1 (total number repeats)		DARC (positive phenotype)	
	Effect	p value	Effect	p value	Effect	p value	Effect	p value	Effect	p value
No biological variables	3.89	0.002		N/S		N/S		N/S	2.25	0.006
LDH	3.13	0.009		N/S		N/S		N/S	2.07	0.007
Bilirubin	2.14	<0.0001		N/S		N/S		N/S	3.21	0.001
Absolute Retic	3.25	0.006		N/S		N/S		N/S	2.82	0.005
WBC	3.19	0.005		N/S		N/S		N/S	2.01	0.008

## Effect of genetic polymorphisms on markers of chronic kidney disease

Impairment in renal function (as defined by an MDRD eGFR  $\leq 60$  see Table 18) was less common than either micro or macro albuminuria in our patient group with 9% having at least one recorded value at this level or below during the study period. It was marginally more common in patients with SCA than patients with Hb SC (9% vs. 7%). This may indicate the multifactorial nature of renal failure with common factors such as hypertension playing a prominent role in pathophysiology.

Interestingly, in contrast to Ashley-Koch *et al* our data showed a significant association with the number of *APOL1* risk alleles and the development of renal impairment (effect 2.40 95% 0.46 – 4.33  $p = 0.02$ ). This remained significant when analysis was additionally corrected for absolute reticulocyte count (coefficient = 1.76 95% CI 0.41 – 3.12  $p = 0.01$ ) and WBC (co-efficient = 2.33 95% CI 0.36 – 4.31  $p = 0.01$ ). All other polymorphisms had no association with the development of renal impairment, including the presence of SCA (summarised in

Table 28).

Table 28: Summary of genotype interactions with haemolytic variables with presence or absence of renal impairment (MDRD eGFR  $\leq 60$ ) as the binary outcome variable.

Biological variable used in analysis	Sickle genotype (effect of SCA)		Alpha genotype (presence of trait)		APOL1 (number of risk alleles)		HMOX1 (total number repeats)		DARC (positive phenotype)	
	Effect	p value	Effect	p value	Effect	p value	Effect	p value	Effect	p value
No biological variables		N/S		N/S	2.40	0.02		N/S		N/S
LDH		N/S		N/S		N/S		N/S		N/S
Bilirubin		N/S		N/S		N/S		N/S		N/S
Absolute Retic		N/S		N/S	1.76	0.01		N/S		N/S
WBC		N/S		N/S	2.33	0.02		N/S		N/S

### 6.3.5 Discussion

We present data on the associations of sickle nephropathy in a well characterised cohort of patients with sickle cell disease and known Duffy phenotype, *APOL1* genotype and *HMOX1* genotype. Our results confirm our previously published findings that Duffy negative patients have lower WBCs. We found no associations of Duffy phenotype, number of *APOL1* risk alleles or number of *HMOX1* promoter repeats with microalbuminuria. However positive Duffy phenotype was associated with the development of macroalbuminuria. In our analysis this appeared to have a similar significance and effect to that of sickle genotype (i.e. presence of SCA). We did not confirm the results of Ashley-Koch *et al* who found an association between macroalbuminuria and *APOL1* risk alleles, however their analysis did not take into account haemolytic variables and WBC or Duffy phenotype. This may be the result of studying a different population, perhaps with different ethnic stratification (although still African-derived) than that found in the United States. *HMOX1* was also not significantly associated with macroalbuminuria.

Also in contrast to Ashley-Koch *et al* we showed a significant association with *APOL1* risk alleles and development of renal impairment (as measured by MDRD eGFR). This remained significant when corrected for reticulocyte and WBC but not for other haemolytic variables. *HMOX1* promoter copy number and Duffy phenotype had no significant association with the development of renal impairment. This positive association with chronic kidney disease mirrors that found in the large US studies of focal segmental glomerulosclerosis and end stage kidney disease populations.

There are limitations to our study. Although treatment factors such as transfusion and hydroxycarbamide were included in our analysis, other common factors effecting renal function (such as hypertension) were not. The numbers of Duffy positive patients are small (10% of our whole cohort), but with the increased significance of the effect of this polymorphism in our expanded cohort (compared to Chapter 5) we conclude that this is an important finding, particularly in the context that a proportion of the cohorts studied world-wide will have a degree of Caucasian admixture indicated by this polymorphism. Whether the effect on renal function seen is a true effect of Duffy phenotype and its effect on WBC (which would be logical considering the role of WBCs in the pathogenesis of SCD, particularly vaso-occlusion) or whether it is a surrogate marker for another Caucasian population derived polymorphism, will

require further study. The differing results from Ashley-Koch *et al* could be explained by the differing methods of analysis and our accounting for other potentially confounding factors. The significant association with MDRD eGFR and *APOL1* risk alleles is similar to what has been found with other non-sickle African derived populations.

In summary, the promoter polymorphism in *HMOX1* had no association with the development of renal dysfunction in our cohort. The Duffy positive phenotype does appear to be significantly associated with the development of macroalbuminuria and should be included in future genetic studies when this is used as an outcome measure. Further work will be needed to show whether the effect of the raised WBC found in this group is causative of the underlying pathology. We have confirmed the results of other groups working in non-sickle populations that *APOL1* is associated with the development of significant renal impairment as measured by MDRD eGFR and this marker could be used to risk stratify patients with SCD, perhaps for more aggressive clinical interventions such as transfusion programmes, commencing hydroxycarbamide or control of hypertension.

## Chapter 7 The Effect of Sickle Cell Disease on the Liver

### 7.1 Introduction

Sickle cell disease (SCD) in the United Kingdom is no longer purely a disease of childhood (Dacie 1960). However, despite the improvements made in patient survival (see Chapter 3), patients with SCD (particularly those with Hb SS and Hb S $\beta^0$ ) still die, on average, 20-25 years earlier than ethnically matched controls (Platt, *et al* 1994), the relatively early mortality due to sickle-related complications which affect multiple organs. Estimates of the prevalence and incidence of complications affecting the liver (generally referred to as “sickle hepatopathy”), and its contribution to morbidity and mortality, vary from series to series, partially due to the heterogeneity of presentations which encompass this clinical entity. Liver disease in SCD appears to present late, by which time fibrosis of the liver is advanced and therefore irreversible. The stage of liver fibrosis is the most important predictor of morbidity and mortality in other chronic liver diseases (Poynard, *et al* 2000a, Poynard, *et al* 2000b) and it would be rational to extend this premise to sickle hepatopathy. Earlier diagnosis of liver involvement would enable earlier intervention and therefore potentially a better outcome for patients.

As in other chronic liver disorders (e.g. hepatitis C) liver biopsy remains the gold standard for diagnosis and monitoring of fibrosis, although novel non-invasive assessment techniques have been validated (mainly in hepatitis C) and are becoming accepted in routine care. Liver biopsy enables direct visualisation and grading of the degree of fibrosis and necrosis and serial samples can be taken over time to monitor progression. However liver biopsy is invasive and cannot be used routinely in SCD for assessing liver damage, due to an increased risk of bleeding, particularly in the acute setting (Zakaria, *et al* 2003) and is not an acceptable screening tool. Instead, an alternative approach, potentially using a combination of investigations, may be required to risk-stratify patients for further investigations, including liver biopsy. Once a population of patients with liver disease has been identified it will then be possible to look for risks for its development, including clinical and genetic factors. This study attempts to 1) characterise the prevalence of sickle liver disease in the King’s College Hospital cohort using a combination of standard liver function tests, routine imaging and novel non-invasive tests, 2) identify associations between sickle liver disease and clinical sub-groups and genetic polymorphisms, and 3) define a tool for screening for liver complications that can be

applied to routine clinical practice so that at risk patients can undergo targeted investigations and monitoring in specialist joint sickle/liver clinics.

#### **7.1.1 Pathogenesis and Staging of Liver Fibrosis**

A unifying pathological process appears to underlie the development of the majority of chronic liver diseases (of which the best characterised is chronic hepatitis C). An inflammatory trigger leads to a series of pathological changes, the final result of which is cirrhosis. Inflammation leads to the transactivation of hepatic stellate cells, leading to increased collagen gene expression and secondary formation of collagen  $\alpha_1$  (Clement, *et al* 1986, Hernandez-Gea and Friedman 2011, Lee, *et al* 1995). This increased collagen deposition leads to the formation of the extracellular matrix which results in disruption of the normal liver architecture, focal and portal fibrosis, and finally the development of cirrhosis.

There are two main mechanisms of liver damage in SCD, which could lead to the unifying pathology mentioned above. The first is secondary to intra-sinusoidal sickling of red blood cells in the liver and secondary engorgement of the Kupffer cells, impeding hepatic blood flow resulting in hepatocyte anoxia and tissue necrosis (Green, *et al* 1953, Rosenblate, *et al* 1970). This tissue death leads to a reduction in bile formation and perturbation of bile flow resulting in intrahepatic cholestasis (Shao and Orringer 1995, Sheehy 1977, Song 1957). Fundamental to this process appears to be ischaemia/reperfusion injury (with an amplified inflammatory response) similar to that found in other conditions (Charlotte, *et al* 1995, Teoh and Farrell 2003), leading to focal areas of necrosis and regeneration. This inflammatory trigger results in the deposition of the extracellular matrix and development of cirrhosis described above.

The second proposed mechanism results from haemolysis. As previously discussed (see 1.2) free plasma Hb, released as a result of intravascular haemolysis, leads to NO consumption and subsequent vasculopathy. Markers of haemolysis correlate with the development of renal dysfunction (Chapter 6), and a similar process might contribute to the pathology of sickle hepatopathy. Work performed in a SCD mouse model highlights the importance of the liver microcirculation in the development of liver disease in this context and the role of vasoconstrictive molecules including endothelin-1 and vasodilatory molecules including NO (Siciliano, *et al* 2011). Thus, speculatively, perturbation of the blood supply via haemolytic



vasculopathy could lead to tissue death and inflammation, again acting as the inflammatory trigger for extracellular matrix deposition.

Co-existent viral hepatitis, providing an additive inflammatory stimulus, could act synergistically with SCD lowering the threshold for the formation of the extracellular matrix and therefore development of liver disease. The reported rates of hepatitis in SCD vary with approximately 3.3% of patients being positive for hepatitis B surface antigen in an American series compared to 27.5% in a series from Saudi Arabia (el-Hazmi and Ramia 1989). The prevalence of chronic hepatitis C appears to be higher with HCV antibody rates of 15-21% being reported {DeVault, 1994 #2078;DeVault, 1994 #2078;Jeannel, 1998 #2081}, although this is linked to the use of unscreened blood transfusion.

It is likely that vaso-occlusive and haemolysis-induced vasculopathies, in combination with the potential complication of viral hepatitis, contribute to the liver complications seen in SCD. This makes the characterisation of sickle hepatopathy and the development of a screening tool more complicated as multiple predisposing factors need to be included.

In other causes of chronic liver disease, clinical importance lies with being able to differentiate between the stages of liver fibrosis (see Table 29) as this correlates strongly with patient life-expectancy. It is reasonable to expect that this is also applicable in SCD. These categories are histological definitions of fibrosis and part of the staging score for the severity of chronic hepatitis. The Knodell histology activity index (HAI) was published in 1981 (Knodell, *et al* 1981), describing the morphological lesions seen in the various stages of chronic hepatitis.

Table 29: The degree of fibrosis and score allocated in the Knodell histology activity index (HAI).

Fibrosis, in combination with other histological factors (periportal, bridging and focal necrosis and portal inflammation) gives an overall score for necrosis, inflammation and fibrosis in the liver. This gives the severity of the histological damage and is used as a prognostic marker

Degree of fibrosis	Score
No fibrosis	0
Fibrous portal expansion	1
Bridging fibrosis (portal-portal or portal-central linkage)	2
Cirrhosis	3

It has undergone several modifications over time; for example, the Scheuer system (Scheuer 1991) assigns 5 grades of fibrosis and the Ishak Modified HAI (Ishak, *et al* 1995) assigns 7 grades to the histological fibrotic changes. The key studies used to ascertain the ranges of novel non-invasive tests discussed in 7.1.7 used the Ishak Modified HAI (see Table 30).

Table 30: The histological characterisation of fibrosis using the Ishak Modified HAI with clinical correlation with the severity of the fibrosis.

Architectural changes, Fibrosis and Cirrhosis	Score	Degree of severity of fibrosis
No fibrosis	0	Mild
Fibrous expansion of some portal areas, with or without short fibrous septa	1	
Fibrous expansion of most portal areas, with or without short fibrous septa	2	Moderate
Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging	3	
Fibrous expansion of portal areas with marked bridging (P-P) and portal-central (P-C)	4	Severe
Marked bridging (P-P and P-C) with occasional nodules (incomplete cirrhosis)	5	
Cirrhosis (probable or definite)	6	

### 7.1.2 The Role of Iron in Liver Dysfunction in Sickle Cell Disease

Therapeutic interventions can have an impact upon the development of liver disease in SCD. Currently, the two main treatment options in SCD are hydroxycarbamide and blood transfusion. Transfusion is increasingly used in SCD in both the acute and chronic settings, to prevent and treat the complications of this disorder, and this can result in iron overload, predominantly in the liver (Adamkiewicz, *et al* 2009, Ballas 2001, Drasar, *et al* 2011). A complication of long term iron overload is liver fibrosis and cirrhosis. Fibrosis results, in part, from increased levels of non-transferrin bound iron which has a high propensity to induce reactive oxygen species and cause cellular damage and also deplete NO levels leading to vasoconstriction and endothelial dysfunction.

### 7.1.3 Clinical Presentations of Liver Complications in Sickle Cell Disease

“Sickle hepatopathy” is an umbrella term for both acute and chronic sickle-related complications affecting the liver. This clinical heterogeneity has led to problems in ascertaining the prevalence

and incidence of liver involvement in SCD. This needs to be understood prior to the creation of a screening tool and performing genetic and clinical association studies, particularly in the selection of which test results are included in any definitions.

Acute liver involvement consists of varied, but clearly defined, clinical presentations. Although acute liver involvement can be very dramatic with an extremely unwell patient, appropriate intervention e.g. antibiotics or blood transfusion can result in a swift resolution of symptoms. *Gallstones* are common in patients with SCD and this can result in an acute transaminase rise during episodes of acute cholecystitis, usually accompanied by right upper quadrant pain and sometimes fever. "*Hepatic crisis*" with *intra-hepatic cholangiopathy* can accompany a generalised vaso-occlusive episode, secondary to acute vaso-occlusive events in the liver. *Hepatic sequestration* is one of the most dramatic liver complications of SCD, during which large volumes of blood are trapped in the liver resulting in acute anaemia and hypovolaemia although the liver dysfunction may be mild. It is typically a complication of childhood. The diagnosis is defined by a significant decrease in Hb from baseline, associated with tender liver enlargement (Ahn, *et al* 2005, Buchanan and Glader 1977). *Acute intrahepatic cholestasis* results from hepatocyte anoxia causing a reduction in bile formation and secondary disturbed bile flow within canaliculi (Ballas, *et al* 2010, Banerjee, *et al* 2001, Berry, *et al* 2007).

The chronic manifestations of sickle hepatopathy are less clearly defined leading to delays in intervention and therefore worse outcomes for patients. Patients can present acutely with decompensation of an underlying chronic problem or be diagnosed as the result of routine monitoring in clinic. Data from Prof Thein and the liver unit at King's College Hospital (KCH) has defined six main clinical sub-groups of chronic liver disease based on a cohort of patients with SCD referred for specialist evaluation from the KCH liver unit over a 6 year period (from 1999-2005) (Berry, *et al* 2007). The clinical, laboratory, radiological and histological features of the cohort (38 patients) were assessed. The most common sub-group was *chronic sickle hepatopathy*, which was defined as a state of chronic sequestration, with liver enlargement but modest derangements in liver function tests. *Acute decompensation of pre-existing cirrhosis* was the second most frequent sub-group and had the worst outcome with all the affected patients dying. *Iron-overload without cirrhosis* (as defined by siderosis and fibrosis on liver biopsy) was also commonly found among sickle cell patients referred to the liver unit with evidence of chronic liver disease. *Mechanical biliary obstruction, cholangiopathy* (secondary to

primary sclerosing cholangitis) and *venous outflow obstruction* were the remaining sub-groups. Mortality was high in the cohort with chronic liver disease, 29% (11/38) of patients dying within the study period. This emphasises the importance of risk stratification and early intervention in this patient group.

#### **7.1.4 Liver Complications as a Cause of Death**

Just as the role of liver disease in sickle cell morbidity may be underestimated, similarly there is little data on its contribution to mortality of patients with SCD. The available information is taken from several case series focusing on all cause mortality in SCD. Platt's 1994 paper defined three of 209 deaths studied (1%) as being directly related to liver dysfunction (including hepatitis) (Platt, *et al* 1994) and in 2005 Powars *et al* found that eight of 232 deaths studied (3%) had liver involvement as a direct cause of death (Powars, *et al* 2005). The following year Darbari *et al* found that 11.3% (16/141) of all deaths studied had cirrhosis as direct cause of death, linked strongly to iron overload (Darbari, *et al* 2006). All these studies have the same limitations: that they are based on small datasets in a group with complex presentations with the potential for multiple organs being affected and are therefore susceptible to a bias in patient selection. They do however show that, although liver disease as a final cause of death is relatively uncommon, it does still appear to contribute in a significant manner towards mortality and morbidity in SCD, and is thus worthy of further investigation.

#### **7.1.5 Treatments**

Commencing early treatment appears to be the key to treating acute liver complications and therefore avoiding chronic liver dysfunction in SCD. There is evidence from the acute setting that prompt exchange transfusion can reverse acute intrahepatic cholestasis in SCD (Ahn, *et al* 2005, Brunetta, *et al* 2011, Sheehy, *et al* 1980, Stephan, *et al* 1995) by reducing HbS% and therefore polymerisation and sickling. It is not unreasonable to extend this premise to the chronic setting with the aim of reducing further liver insults, particularly as once cirrhosis is established (as in other causes of liver failure) there are few treatment options available. Management is centred around supportive care with regular paracentesis, banding of varices to prevent acute bleeding episodes and support with coagulation factors. However if the

diagnosis is made prior to the cirrhotic stage, then interventions including exchange transfusion and aggressive iron chelation (where iron overload is present) could be initiated, with the aim of slowing or potentially reversing progression from fibrosis to cirrhosis.

Transfusion is the main treatment used in both acute and chronic liver failure in SCD. Exchange rather than top-up transfusion is recommended when possible to minimise iron loading, as hepatic siderosis will have an impact on the development of liver disease (see 7.1.2).

Although hydroxycarbamide has been highly successful in decreasing the frequency of painful episodes and acute chest syndrome in patients with SCD (Charache, *et al* 1996, Wang, *et al* 2011) it appears to have no effect on the development of acute hepatic sequestration. Further work on hydroxycarbamide is warranted to investigate the long term effect on chronic liver dysfunction.

Liver transplantation is rarely carried out in patients with SCD; there are a total of 23 case reports in the literature (Baichi, *et al* 2005, Berry, *et al* 2007, Delis, *et al* 2006, Emre, *et al* 2000, Gardner, *et al* 2014, Gilli, *et al* 2002, Greenberg, *et al* 2009, Hurtova, *et al* 2011, Kindscher, *et al* 1995, Lang, *et al* 1995, Lerut, *et al* 1999, Mekeel, *et al* 2007, Perini, *et al* 2010, Ross, *et al* 2002, van den Hazel, *et al* 2003). The largest adult cohort was published by Hurtova *et al* and gives their experience of transplanting 6 adults with SCD over a 17 year period, 4 of whom had co-existent hepatitis B or C and 5 of whom had iron overload. Four of the patients died during follow up with a mean survival of 4 years post liver transplant; although this is an improvement in life expectancy, it is still less than would be expected when comparing liver transplant outcomes for non-sickle indications. The paediatric experience of liver transplantation is also small, but 5 year survival was 100% in the reported cohort (Mekeel, *et al* 2007). The main indication for transplantation appears to be intrahepatic cholestasis with and without co-existent hepatitis C. Specific suggestions for improvement of outcomes and long-term post-transplant survival include the placement of patients on long-term transfusion programmes both before and after transplantation surgery (aiming for a Haemoglobin S% of less than 30%), attention to peri-operative thrombosis risk, immunosuppression-related infections, and management of neurological toxicity related to the immunosuppression required.

### **7.1.6 Current Methods of Assessing Liver Function**

As previously discussed, the presentations of liver dysfunction are heterogenous. This makes its early diagnosis and the development of a screening tool complicated, particularly as the standard test of liver biopsy is relatively contraindicated in this patient group. It is also unclear as to whether standard markers of poor prognosis (reduced synthetic function, increased liver size) also apply to the SCD population. Current methods for delineating liver damage include laboratory tests (serum transaminases, markers of synthetic function, ammonia levels), and traditional imaging techniques (ultrasound, computerised tomography (CT) and magnetic resonance imaging (MRI)).

#### **7.1.6.1 Liver Function Tests**

Serum liver function tests at King's College Hospital are measured using the Advia Centaur® XP immunoassay system (Siemens, Surrey, UK) and include markers of necrosis and liver damage (liver enzymes) as well the synthetic function of the liver (serum albumin). They have the advantage of being widely available, relatively cheap to perform and minimally invasive. They are also easily repeatable and thus longitudinal data is available. These are all strong reasons for their use in the diagnosis of liver disease and in any screening tool developed.

There are four routinely measured liver enzymes which are found within hepatocytes and are therefore released as a result of liver damage. Unfortunately they are not specific to the liver and are found in a variety of other tissues (see below). They are aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT). AST is an enzyme that catalyses aspartate and  $\alpha$ -ketoglutarate to oxaloacetate and glutamate. It is found in liver, skeletal muscle, red blood cells, kidney, brain and cardiac muscle. It is therefore not specific for damage to liver cells, particularly in SCD where chronic haemolysis could contribute a significant amount to a raised AST level. Different isoforms of AST are released from different tissues, however, isoform subtyping is not routinely performed in clinical practice, making this differentiation difficult. ALT is an enzyme that catalyses the transfer of an amino group from alanine to  $\alpha$ -ketoglutarate, the resultant products being pyruvate and glutamate. It is more specific for hepatocellular damage than AST, but shows marked diurnal variation. ALP is an enzyme found throughout the whole body and is

responsible for the removal of phosphate groups from a multitude of proteins. It is found in higher concentrations in the intestine, bone, liver and kidney and is raised in biliary obstruction. However bone infarction/infection can also cause elevated levels (of particular significance in SCD during acute episodes). GGT is an enzyme again found in the cell membranes of multiple tissues and is involved in the transfer of amino acids across the cell membrane as well as leukotriene metabolism. Like ALP, raised levels of GGT are found in cholestasis, but it is not raised during bone damage. It can however be raised in congestive cardiac failure and by specific medications (including non-steroidal anti-inflammatories) and alcohol use (Green, *et al* 1953, Rosenblate, *et al* 1970, Song 1957, West, *et al* 1992).

One of the key features of sickle cell intrahepatic cholestasis is conjugated hyperbilirubinaemia. The conjugated aspect is important, differentiating it from the raised unconjugated hyperbilirubinaemia seen in haemolytic episodes of sickle cell disease. Although most commonly an acute presentation, a chronically raised conjugated bilirubin could be an important indicator of a more chronic cholestatic picture.

Equally important is the synthetic function of the liver, the most commonly used measure being serum albumin. Unfortunately the levels of this protein only start to fall when the liver functional reserve has reached critical levels. Levels of albumin can also fall in acute illness, for example sepsis. Another measure of the liver synthetic function is the International Normalised Ratio (or INR) which is a reflection of production of vitamin K dependant coagulation factors that are synthesised in the liver.

The normal ranges for the laboratory at King's College Hospital are shown in Table 31. Also shown are one-and-a-half times and twice the upper limit of normal. These values are commonly used during pharmaceutical trials as markers of "true" abnormal values and reasons for stopping the medication to be tested.

Table 31: Serum liver function tests with normal ranges at King's College Hospital and limits for abnormal values.

Upper limit of normal multiples chosen due to common usage during drug trials and medication usage as unacceptably high values, indicative of potential liver dysfunction.

Serum liver function test	Normal range	1.5 x upper limit of normal	2 x upper limit of normal
Alanine transaminase (ALT)	5 – 55 IU/L	≥ 82.5 IU/L	≥ 110 IU/L
Albumin	35 – 50 g/L	N/A	N/A
Aspartate transaminase (AST)	10 – 50 IU/L	≥ 75 IU/L	≥ 100 IU/L
Alkaline phosphatase (ALP)	20 – 130 IU/L	≥ 195 IU/L	≥ 260 IU/L
Gamma-glutamyl transpeptidase (GGT)	1 – 55 IU/L	≥ 82.5 IU/L	≥ 110 IU/L
Conjugated bilirubin	<4 µg/L	≥ 6 µg/L	≥ 8 µg/L

#### 7.1.6.2 Liver Imaging

Routine liver imaging is widely available, reproducible and gives information about the size and structure of the liver, the accuracy and degree of detail being dependent on the modality chosen. However radiological findings in chronic sickle liver disease are not clearly defined. Hepatomegaly is the most common finding in chronic sickle liver disease (Berry, *et al* 2007); this is an abnormal enlargement of liver size defined by a volumetric change. The body mass of the patient has been reported to correlate with the volume of the liver (Andersen, *et al* 2000, Leung, *et al* 1986, Linguraru, *et al* 2012, Wynne, *et al* 1989) which is important in normalization of values, enabling comparisons between subjects and different cohorts of patients. Hepatic size is also an important biomarker for assessing the effect of a variety of conditions on the liver (Elstein, *et al* 1997, Sekiyama, *et al* 1994, Zoli, *et al* 1991). Predictably, hepatic size estimates by physicians using palpation and percussion are approximate (Sherlock 1981) and adequate for diagnosing only massive hepatomegaly cases (Castell, *et al* 1969). Currently the most accurate method of measuring liver size is Magnetic Resonance Imaging (MRI).

Different radiological investigations have different strengths and therefore applications. Ultrasound is widely available, does not involve exposure to radiation and requires minimal equipment. However it is highly operator specific and images cannot be reviewed at a later date by an independent operator. It is useful in investigating causes of liver dysfunction including malignancy, vessel obstruction and gallstones as well as providing an estimation of liver size (Madani, *et al* 2007). Computerised Tomography (CT) can be used to delineate



abnormalities in hepatic size and for characterisation of focal lesions including abscesses and necrosis (Magid, *et al* 1984). CT scans result in exposure to radiation and are therefore not recommended for routine screening in non-malignant conditions. MRI has the advantage over CT of not involving exposure to radiation. It is also the most accurate method of assessing hepatic size and providing a more detailed picture of the liver parenchyma. The images from both CT and MRI scans can be reviewed by other trained operators at later dates to provide further information. Spin density projection assisted R2-MRI (FerriScan®, Resonance Health Limited and Resonance Health Analysis Services Pty Limited, Claremont, Australia) (St Pierre, *et al* 2005) is a well validated method of assessing iron loading in the liver, thus avoiding the need for liver biopsy.

#### **7.1.6.3 Liver Biopsy**

The gold standard for assessment of the diagnosis and monitoring of liver fibrosis is liver biopsy. The reasons why this investigation cannot be used routinely in SCD have been outlined in 7.1.

#### **7.1.7 Specialist Diagnostics**

As previously discussed the extent of liver disease in the sickle cell population is not well understood. Liver biopsies in sickle cell disease appear to carry excess risks of bleeding (Berry, *et al* 2007, Zakaria, *et al* 2003) particularly in the acute setting and due to their invasive nature are not appropriate as a screening tool. In other chronic liver diseases (e.g. Hepatitis C) there has also been a move away from liver biopsy, towards non-invasive tests to assess the degree of fibrosis particularly given the key role of severity of fibrosis in disease stratification. The 2 main examples of this, currently used routinely in hepatitis C are transient elastography (FibroScan® Echosens, France) and the Enhanced Liver Fibrosis Score (ELF™ score, iQUR, London). Both of these tests are available at King's College Hospital and were used a) to assess the degree of fibrosis in our SCD population and b) as surrogates for degree of fibrosis (which would have traditionally been diagnosed using liver biopsy) when constructing the screening tool.

### 7.1.7.1 Transient Elastography

Transient elastography (FibroScan® Echosens, France) is a useful method to detect fibrosis using ultrasound and low frequency (50Hz) elastic waves with a propagation velocity directly related to the stiffness of the liver. It is routinely used as an alternative to liver biopsy in hepatitis C, correlates well with the existing gold standard of liver biopsy and is extremely reproducible (Boursier, *et al* 2008, Castera, *et al* 2005). Transient elastography has certainly helped as a screening tool; only patients with readings indicative of significant fibrosis proceed to a liver biopsy.

Transient elastography is an ultrasound based technique with vibrations being transmitted to the skin via a probe, inducing a shear wave which propagates into the liver. A pulse–echo acquisition is used to measure the wave’s velocity which is proportional to tissue stiffness. FibroScan® was the subject of a recent systematic review which concluded that whilst FibroScan® has excellent diagnostic capability across different liver diseases for cirrhosis (i.e. advanced fibrosis) there was some variability in accuracy for diagnosis of lesser degrees of fibrosis (Friedrich-Rust, *et al* 2008). The suggested diagnostic ranges from this analysis are shown in Table 32. Also shown are the diagnostic ranges used in clinical practice by the liver department at King’s College Hospital.

Table 32: Ranges and clinical correlation with fibrosis based on King’s College Hospital liver department guidelines and published meta-analysis (Friedrich-Rust *et al*)

	Severity of fibrosis (based on Ishak modified HAI score) (kPa)		
	None/mild	Moderate	Severe
King’s guidelines	0 to 7	7.1 to 12	≥ 12.1
Friedrich–Rust <i>et al</i> (Friedrich-Rust, <i>et al</i> 2008)	0 to 7.65	7.66 to 13.00	≥ 13.01

FibroScan® has been used previously in SCD by two groups. Voskaridou *et al* have presented data on 110 patients with mixed genotypes (Hb SS and Hb Sβthal) (Voskaridou, *et al* 2010). They found correlations with liver MRI T2\* values (measurement of iron loading in the liver), serum ferritin and number of transfusions, as well as with markers of haemolysis. Strong correlations were also found with markers of liver function. Koh *et al* (Koh, *et al* 2013) have

recently published data on transient elastography, showing a positive correlation with liver biopsy fibrosis in small cohort of patients (using the Ishak score). They also showed an increase in transient elastography values “intra-patient” during acute painful episodes. Transient elastography has also been used to assess iron-related hepatic fibrosis in haemachromatosis (Adhoute, *et al* 2008).

#### 7.1.7.2 Enhanced Liver Fibrosis Score

The Enhanced Liver Fibrosis Score (ELF™ score, iQUR, London) was devised by Prof William Rosenberg’s research group (based at the Royal Free Hospital, London). It is also used to assess the degree of fibrosis in patients with Hepatitis C, although is not commonly used at King’s College Hospital.

Whereas transient elastography examines the amount of existing fibrosis (or deposited extracellular matrix) the ELF™ score examines the balance between matrix deposition and degradation i.e. the current activity of the fibrotic process. ELF™ score combines serum levels of hyaluronic acid, amino-terminal propeptide-of-type-III-collagen (PIIINP), and tissue-inhibitor of matrix-metalloproteinase-1 (TIMP1) and has an AUROC of 0.8 (Friedrich-Rust, *et al* 2010, Parkes, *et al* 2010, Rosenberg, *et al* 2004, Trepo, *et al* 2011). These three proteins, which are single markers of hepatic matrix metabolism, are then analysed using a patented formula to calculate a score for fibrosis, based on the Ishak modified scoring system (0 = normal, 1 = moderate and 2 = severe). Different groups have defined different scoring ranges, depending on the end point considered: established fibrosis/cirrhosis (Lichtin角度 *et al*)(Lichtin角度, *et al* 2013) or likelihood of developing cirrhosis (Parkes *et al*)(Parkes, *et al* 2010).

Table 33: Published ranges for extended liver fibrosis score and clinical correlations

ELF™ score used	Severity of fibrosis (based on Ishak modified HAI score) or risk of developing fibrosis (Parkes <i>et al</i> )		
	None/mild	Moderate	Severe
Manufacturers’ ranges (iQUR™)	≤ 7.7	7.8 to 9.7	≥ 9.8
Lichtin角度 <i>et al</i> (Lichtin角度, <i>et al</i> 2013)	≤ 9.7	9.8 to 11.2	≥ 11.3
Parkes <i>et al</i> (Parkes, <i>et al</i> 2010)	≤ 8.34	8.35 to 10.42	≥ 10.43

### 7.1.8 Genetic predictors – HMOX-1

As discussed in Chapter 1 genetic variance has been shown to alter the severity and development of complications associated with SCD. Having used the methods described in 7.1.6 and 7.1.7 to ascertain the prevalence of liver disease in our SCD cohort we could then proceed to look for genetic associations. Understanding genetic predictors for the development of liver dysfunction could enable the tailoring of monitoring programmes on a patient by patient basis.

We speculate that *HMOX-1* could be a candidate gene worthy of exploration as a predisposing factor in the development of liver disease in SCD. Heme oxygenase is a rate-limiting enzyme in heme degradation, leading to the generation of free iron, biliverdin, and carbon monoxide. Induction of *HMOX-1* transcription in SCD is implicated in the antioxidant defence mechanism and can modulate vascular function (Bains, *et al* 2010, Belcher, *et al* 2010, Siciliano, *et al* 2011). Therefore decreased transcription may result in reduced nitric oxide availability and endothelial dysfunction and vasculopathy.

The transcription of *HMOX-1* appears to be affected by the number of GT repeats (GT<sub>22-42</sub>) in its promoter region, with peaks at 23 and 30 repeats (see Figure 21). Cell lines containing fewer repeats appear to have increased transcriptional activity under stress, leading to higher heme oxygenase enzyme activity (Hirai, *et al* 2003).

Work in a sickle mouse model by Belcher *et al* has shown the potential protective role of *HMOX-1* in sickle hepatopathy (Belcher, *et al* 2010). SCD patients and mice are known to have elevated levels of hemoxygenase-1 in response to chronic haemolysis, however this may be insufficient to cope with the increased oxidative demands placed upon the system by the increased heme released during red cell breakdown. The purpose of the study was to use gene therapy to increase the amount of heme oxygenase production in a sickle mouse model and assess the effects on resistance to hypoxia induced damage. S+S-Antilles mice (a moderately severe sickle mouse model) were inoculated with a wild-type or a null-type rat *HMOX-1* transgene. Using dorsal skin fold chambers the group could observe that vascular stasis under hypoxic conditions was inhibited in the *HMOX-1* wild-type (control) group compared to the *HMOX-1* null group. It was also noted that expression of vascular adhesion molecules were decreased. Since polymorphisms in the *HMOX-1* gene modulate its expression, it is possible

that the *HMOX-1* genotype has an influence on liver complications in SCD. Speculatively, the *HMOX-1* genotype could be incorporated into any risk stratification tool developed.

#### **7.1.9 Objectives**

Liver disease is a poorly understood complication of SCD. Our objectives to improve our understanding of the development of this complication and to identify potential predictors include:

- a) To evaluate standard laboratory tests and imaging in combination with novel non-invasive techniques as predictors of liver dysfunction adult patients with SCD**
- b) To evaluate genetic factors (*HMOX1*) and effect of iron loading as predictors for the development of liver dysfunction in adult patients with SCD.**
- c) To use the above data to develop a screening tool for the early diagnosis of liver dysfunction in SCD.**

## **7.2 Methods**

### **7.2.1 Collection of Biological Data**

Biological data including haemoglobin (Hb), lactate dehydrogenase (LDH), total white blood cell count (WBC), neutrophil count, HbF, reticulocyte count, ferritin, creatinine, urine albumin creatinine ratio (ACR), cystatin C and erythropoietin levels were collected from routine blood results in steady state clinic during a 12 year period from January 1<sup>st</sup> 2001 to December 31<sup>st</sup> 2012. Markers of liver function including aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT) and albumin were also collected from the electronic patient record (EPR). Normal ranges and definitions of abnormal results are shown in Table 31. In SCD, blood test results outside the standard normal ranges (particularly in SCA) are common. In relation to serum liver function tests, the clinical

significance of this (particularly in view of the lack of specificity outlined in 7.1.6.1) is unknown and therefore a higher level may add information and be of improved specificity in detecting sickle liver disease. We therefore also defined once-and-a-half and twice the upper limit of normal as alternative limits of abnormality, these are commonly used in clinical trials as levels at which trial medications should be stopped and are therefore potentially a more solid definition of abnormality. Age of patient at the time of sampling was recorded. Clinical data regarding hydroxycarbamide and transfusion therapy was also recorded for each sample.

### **7.2.2 Genetic Data**

The GT repeats in the *HMOX1* promoter region were genotyped; as described in 6.3.2.3. The number of GT repeats correlates inversely with the efficiency of *HMOX-1* transcription (see 7.1.8).

### **7.2.3 Transient Elastography and Enhanced Liver Fibrosis Score**

These scans were performed by Dr Emer Fitzpatrick (10 scans) and myself using a FibroScan® 502 (204 scans). Training in use of the FibroScan® machine was provided by Echosens™. Patients were excluded from the scan if they were pregnant, had an active implantable medical device or ascites. The scans were performed when the patient was in steady-state, with no painful episodes requiring an increase in regular analgesia or hospital admission for the preceding 6 weeks. Ten separate measurements were taken from each patient as per Echosens™ guidance on use. Probe size was chosen according to patient body mass index. Results were recorded as a continuous variable in kilopascals (kPa). Results were only accepted if 90% or over of the readings were valid. The intraquartile range also had to be less than 0.7 for the result to be included in the analysis. 10 patients underwent 2 assessments to assess operator variability, no significant difference was found between measurements. Serum samples for enhanced liver fibrosis (ELF) score testing were collected within 3 months of the FibroScan® being performed (the majority on the same day) into serum separator tubes from BD. The whole blood samples were allowed to clot for a minimum of 30 minutes and then centrifuged at 1300g for 10 minutes at room temperature. Serum was frozen in 2ml aliquots at -80°C. ELF testing was performed using a patented ELISA technique (AVIDA Centaur Systems)

by Tracy Chapman (Department of Biochemistry, King's College Hospital, London) and ELF score calculated using a patented formula.

#### **7.2.4 Statistical Analysis**

Data was manipulated in Excel (Microsoft, Seattle) and SPSS (IBM, New York). Variables were log transformed where appropriate to obtain a normal distribution. The [GT] repeats in the *HMOX1* promoter region were analysed as a continuous variable. FibroScan® and ELF™ measurements were analysed as categorical variables, grouped according to the classifications outlined above. The relevant analyses used are described in each section. As discussed above (7.1.6.3) liver biopsy is not performed in SCD patients and therefore for the screening tool abnormal FibroScan® and abnormal ELF score™ were used as the dependant (outcome) variables.

### **7.3 Results**

The study cohort consisted of 584 patients of mixed sickle genotypes who had steady-state blood tests available from a 6 year period from the 1<sup>st</sup> of January 2007 to the 31<sup>st</sup> of December 2012. 246 (42%) of the group were male, the remainder (338, 58%) were female. Ages ranged from 17 to 82 years at last blood sample (mean 33 years). 359 (61%) were Hb SS, 194 (33%) Hb SC, 27 (5%) Hb Sβ<sup>+</sup>, 3 (1%) Hb Sβ<sup>0</sup> and 1 patient, Hb SHPFH (<1%). For the purposes of this study, patients with Hb SS and Hb Sβ<sup>0</sup> were analysed as a group (sickle cell anaemia, SCA).

#### **7.3.1 Hepatitis Serology and Alcohol Usage**

Two patients (1 SCA and 1 Hb SC) had positive hepatitis C RNA detected. Only 1 patient (with SCA) had active ongoing hepatitis B. All three were under long-term follow up by the liver department at King's College Hospital. Mean alcohol intake amongst our patient group was 1 unit per week (range 0 – 20). The patients with hepatitis C and B were excluded from further analyses. Due to alcohol consumption being low in our population it was not included as a variable in further analyses.

### 7.3.2 Serum Liver Function Tests

Steady state serum liver function tests (sLFTs) were available on the whole patient group and sub-groups, apart from ALT which was introduced as a test half way through the study period. Patients with SCA had significantly lower mean albumin, and a significantly higher AST, ALP, GGT and conjugated bilirubin than Hb SC patients (see Table 34).

Table 34: Serum liver function tests in whole cohort and SCA and Hb SC sub-groups showing effect of sickle genotype on mean values. The p value refers to the difference in mean value between the SCA and Hb SC groups (NR = normal range ULN = upper limit of normal)

Serum liver function test	Whole cohort	Sickle cell anaemia	Hb SC	p value
<b>Albumin (n)</b> <b>NR (35-50 g/L)</b>	584	362	194	
Range (mean)	27 – 53 (43.4)	27 – 50 (43.1)	30 – 53 (43.8)	<b>0.005</b>
Abnormal (%)	15 (2)	13 (3)	2 (1)	
Albumin <30 (%)	5 (<1)	4 (<1)	1 (<1)	
<b>ALT (n)</b> <b>NR (5 – 55 IU/L)</b>	320	204	99	
Range (mean)	2 – 111 (23.3)	2 – 65 (23.4)	8 – 111 (23.1)	N/S
Abnormal (%)	4 (1)	2 (1)	2 (2)	
≥ 1.5 x ULN ≥ 82.5 IU/L (%)	0	0	0	
≥ 2 x ULN ≥ 110 IU/L (%)	0	0	0	
<b>AST (n)</b> <b>NR (10-50 IU/L)</b>	584	362	194	
Range (mean)	14 – 397 (37.2)	14 – 397 (42.7)	15 – 120 (27.9)	<b>&lt;0.0001</b>
Abnormal (%)	93 (16)	86 (24)	7 (4)	
≥ 1.5 x ULN ≥ 75 IU/L (%)	11 (2)	10 (3)	1 (<1)	
≥ 2 x ULN ≥ 100 IU/L (%)	5 (1)	2 (<1)	1 (<1)	
<b>ALP (n)</b> <b>(NR 20-130 IU/L)</b>	584	362	194	
Range (mean)	26 – 714 (91.3)	26 – 428 (98.7)	33 – 714 (77.3)	<b>&lt;0.0001</b>
Abnormal > 130 (%)	73 (13)	62 (17)	8 (4)	
≥1.5 x ULN ≥ 195 IU/L (%)	18 (3)	17 (5)	1 (<1)	
≥2 x ULN ≥ 260 IU/L (%)	3 (<1)	2 (1)	1 (<1)	
<b>GGT (n)</b> <b>(NR 1-55 IU/L)</b>	584	362	194	
Range (mean)	3 – 596 (50)	3 – 596 (58)	4 – 268 (36)	<b>&lt;0.0001</b>
Abnormal > 50 (%)	165 (28)	124 (34)	36 (19)	
≥1.5 x ULN ≥ 82.5 IU/L (%)	90 (15)	72 (20)	18 (9)	
≥2 x ULN ≥ 110 IU/L (%)	59 (10)	49 (14)	10 (5)	
<b>Conjugated bilirubin</b> <b>(NR &lt;4 µg/L)</b>	484	307	156	
Range (mean)	2 – 752 (13.1)	3 -752 (15.9)	2 – 75 (8.7)	<b>0.04</b>
Abnormal > 4 (%)	469 (97)	304 (99)	148 (95)	
≥1.5 x ULN ≥ 6 µg/L (%)	384 (79)	284 (93)	94 (60)	
2 x ULN ≥ 8 µg/L (%)	305 (63)	239 (78)	64 (41)	

. A greater proportion of patients with SCA also had abnormal sLFTs than patients with Hb SC.



The most commonly abnormal sLFT was GGT, with over a third of patients with SCA having an abnormal result. ALT is rarely abnormal in our population (although values were available in only 55% of our cohort). To attempt to add a further layer of discrimination we also examined the proportions of patients with x1.5 and x2 the upper limit of normal (ULN) of the sLFTs, with a view to incorporating these results into our treatment algorithm. Unsurprisingly, GGT remained the most commonly abnormal sLFT in both the SCA and Hb SC populations. No patients had an ALT x1.5 or x2 the ULN (Table 34).

The pattern of sLFT abnormalities can be suggestive as to the underlying pathology, as discussed in 7.1.6.1. Altogether 172 (48%) SCA patients had at least 1 abnormal sLFT; 87 (24%) had only 1 LFT that was abnormal, 61 (17%) had 2 abnormal LFTs, 18 (5%) had 3 abnormal LFTs and 6 (2%) had 4 abnormal LFTs. The patterns of abnormal LFTs in patients with SCA are shown in Figure 22, limited to those patients who had all 5 serum liver function tests. No patients had an isolated raised ALT.

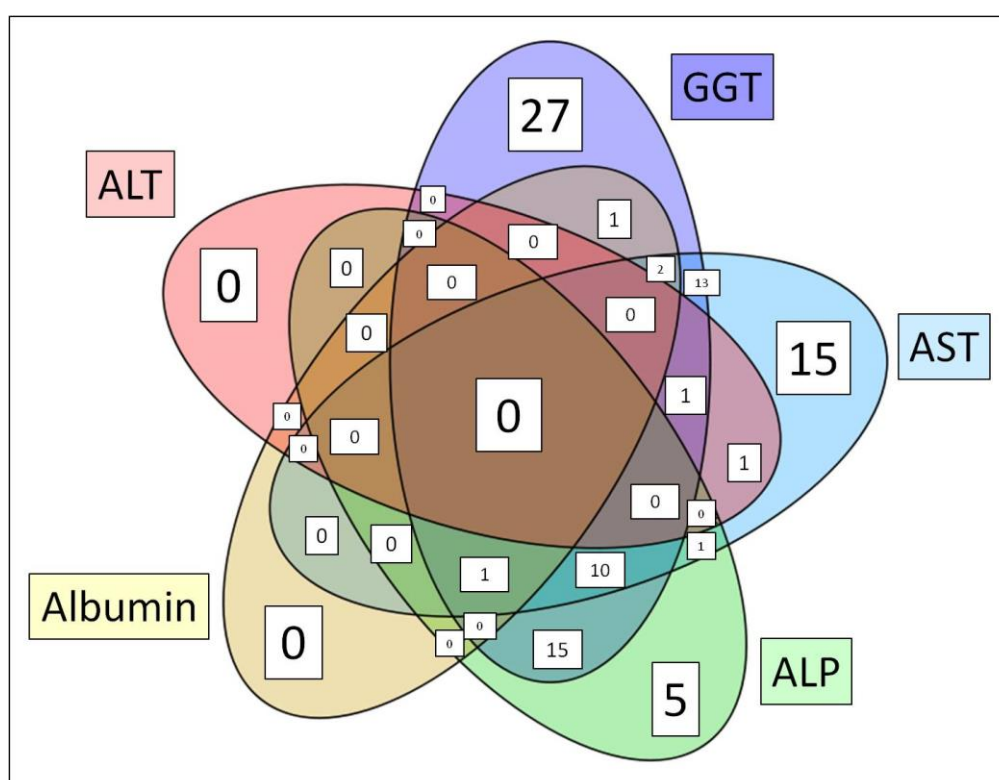


Figure 22: Diagram to show the patterns of liver function abnormalities in the SCA population. This diagram refers to the subgroup of 204 SCA patients who had all 5 LFTs recorded.

### 7.3.3 Transient Elastography

Transient elastography is an easily reproducible test that appears to have good discrimination of severe fibrosis and cirrhosis, however it appears to be less accurate in the diagnosis of early fibrosis. FibroScan® was performed in 214 patients (a subpopulation of the whole cohort). 87 (41 %) were male the remainder (127, 59 %) were female. Age ranged from 17 to 72 (mean 34.6). 146 (68%) patients had SCA, 55 (26%) Hb SC and the remainder Hb S $\beta^+$ . The Hb S $\beta^+$  patients were not included in sub-group analysis due to low numbers. FibroScan® was performed in clinic, when patients were in steady state.

There was a significant correlation between FibroScan™ and age (when corrected for sickle genotype)  $\beta = 0.19$   $p = 0.006$  (see Figure 23). Gender appeared to have an effect on liver stiffness, with men having a higher mean scan result than women (6.74 SD  $\pm$  2.7 vs. 6.00 SD  $\pm$  3.12 kPa), although this result was not significant when corrected for sickle genotype.

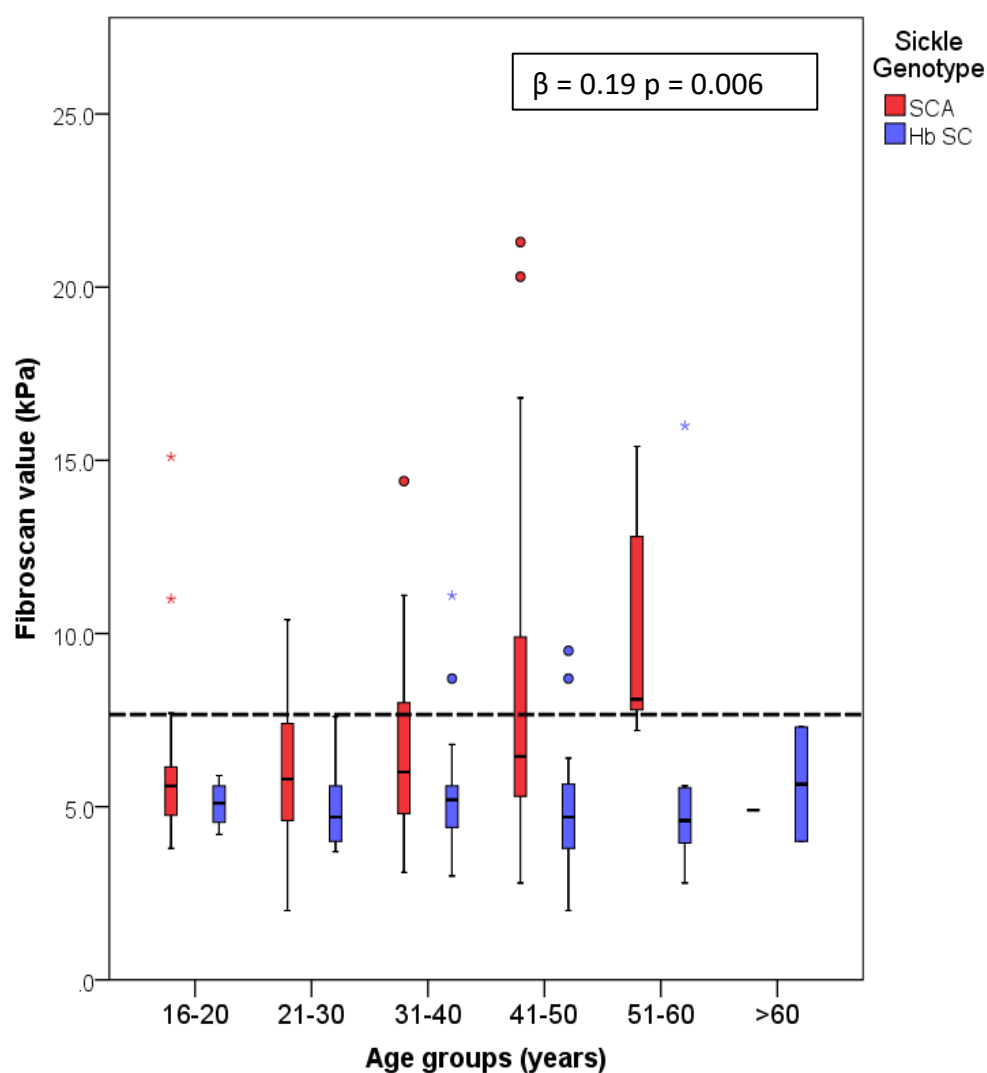


Figure 23: Effect of age and sickle genotype on FibroScan® value.

Area above horizontal limit line indicates abnormal FibroScan® value ( $\geq 7.66$  kPa Friedrich–Rust *et al* (Friedrich-Rust, *et al* 2008)).

As shown in Table 35 and Figure 23, patients with SCA had a significantly higher FibroScan® results than those with Hb SC, reflecting the generally more severe nature of this genotype (6.8 vs. 5.3  $p < 0.0001$ ). 25% (King’s guidelines,  $\geq 7.1$  kPa) and 23% (according to Friedrich-Rust *et al* guidelines,  $\geq 7.66$  kPa) of SCA patients had moderate or severe fibrosis on FibroScan®. FibroScan® value increases with age in SCA patients while the values remain fairly stable with age in Hb SC patients. This is of potential clinical significance given the high morbidity and mortality from liver cirrhosis in the SCD population.

Table 35: The range and mean FibroScan <sup>TM</sup> results for whole cohort and SCA and Hb SC subgroups.

Number and percentage of patients in each group with each separate stage of fibrosis according to King's score and the meta-analysis by Friedrich-Rust *et al* (Friedrich-Rust, *et al* 2008). p value indicates significant difference between the mean FibroScan score for SCA and Hb SC sub-groups

<b>FibroScan score (kPa)</b>	<b>Whole cohort n = 214 (%)</b>	<b>SCA n = 146 (%)</b>	<b>Hb SC n = 55 (%)</b>	<b>p value</b>
<b>Mean (range)</b>	6.3 (2.0 – 23.2)	6.8 (2.0 – 23.2)	5.3 (2.0 – 16.0)	<b>&lt; 0.0001</b>
<b>King's guidelines</b>				
None/mild (0 - 7)	160 (75)	99 (68)	48 (87)	
Moderate (7.1 – 12)	42 (20)	36 (25)	6 (11)	
Severe (≥ 12)	12 (5)	11 (7)	1 (2)	
<b>Friedrich–Rust <i>et al</i> (Friedrich-Rust, <i>et al</i> 2008)</b>				
None/mild (0 – 7.65)	166 (77)	104 (71)	49 (89)	
Moderate (7.66 – 13.00)	38 (18)	33 (23)	5 (9)	
Severe (≥13.01)	10 (5)	9 (6)	1 (2)	

In SCA patients FibroScan <sup>TM</sup> correlated strongly (using linear regression) with serum liver function tests (Albumin R = -0.35, p<0.0001, AST R = 0.44, p<0.0001, ALP R = 0.29, p<0.0001, GGT R = 0.40, p<0.0001, Conjugated bilirubin R = 0.26 p = 0.004). A positive correlation was also found with LDH (R = 0.24 p = 0.004) (Figure 25) and a negative correlation with Hb (R = -0.25, p = 0.002). These results are summarised in Figure 24.

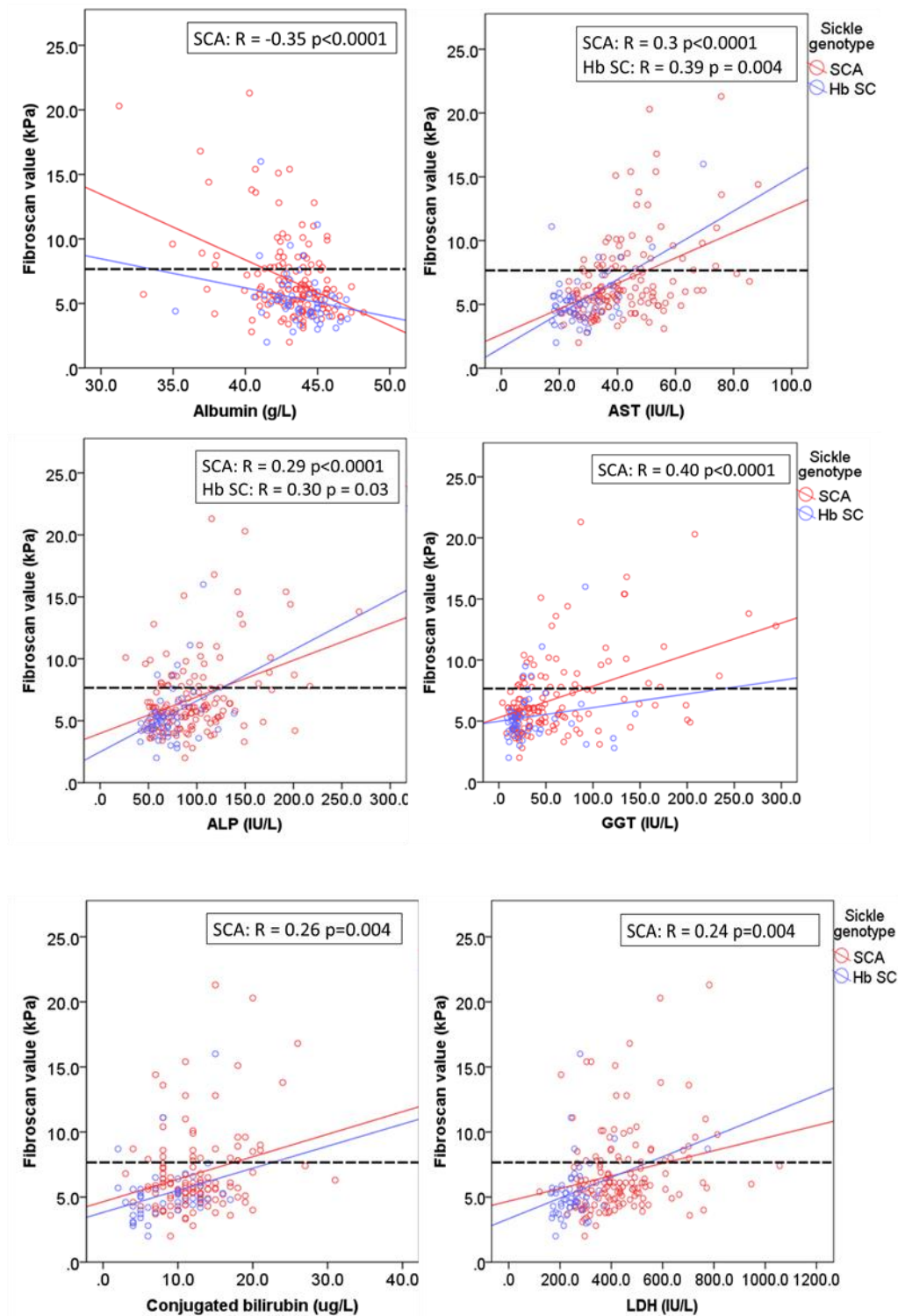


Figure 24: Graphs show the relationship between FibroScan® value and liver function tests.

Significant correlations are seen in SCA with Albumin, AST, ALP, GGT, Conjugated bilirubin, and LDH. In Hb SC the correlations were only significant for AST and ALP. The horizontal reference line indicates an abnormal FibroScan® result ( $\geq 7.66$  kPa Friedrich–Rust *et al* (Friedrich-Rust, *et al* 2008))

No significant correlations were found with other biological variables including HbF levels. When the limit of LDH 550 IU/L (1 standard deviation above our population mean) was used to delineate 2 populations significant differences were found between mean FibroScan results (5.98 kPa in the <550 IU/L group and 8.77 kPa in the  $\geq 550$  IU/L group) with  $p < 0.0001$ , see Figure 25.

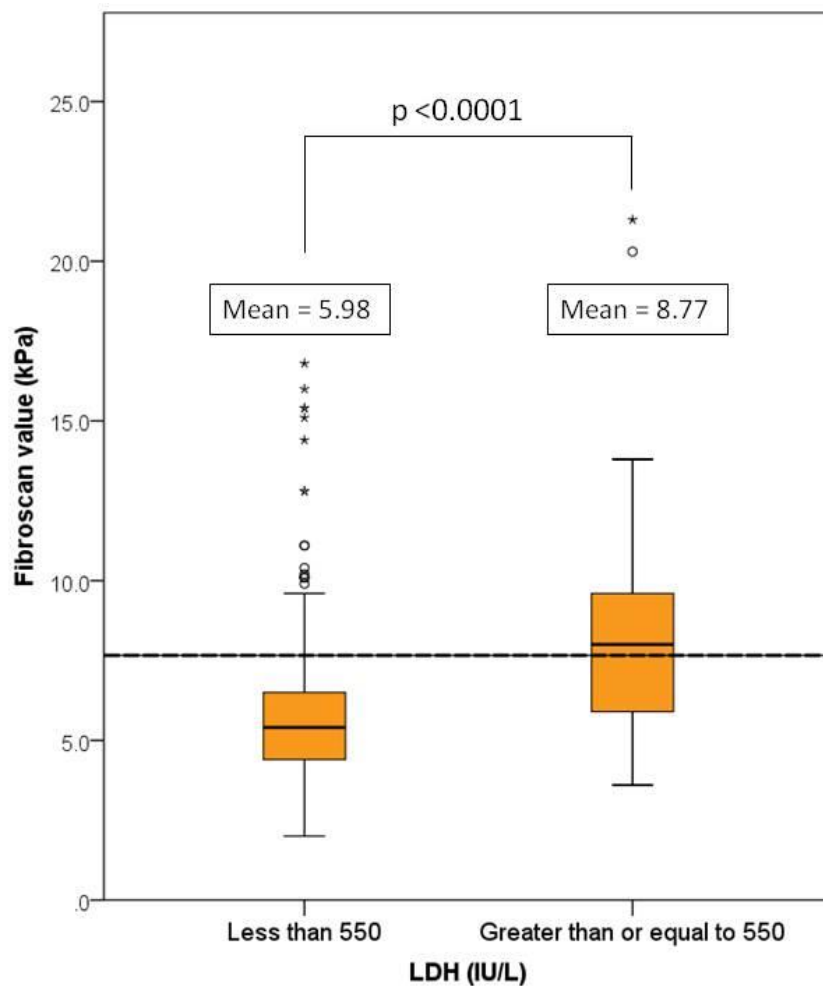


Figure 25: FibroScan ® divided into 2 populations based on LDH value

The horizontal line indicates the presence of an abnormal FibroScan ® value ( $\geq 7.66$  kPa Friedrich–Rust *et al* (Friedrich-Rust, *et al* 2008)).

In the Hb SC group the correlations were weaker and only significant for AST ( $R = 0.39$ ,  $p = 0.004$ ) and ALP ( $R = 0.30$ ,  $p = 0.03$ ) (see Figure 24). However a significant association was

seen between WBC ( $R = 0.39$ ,  $p = 0.02$ ), reticulocyte count ( $R = 0.35$ ,  $p = 0.01$ ) and FibroScan®.

ELF score correlated with FibroScan® but the  $R$  was only 0.37 ( $p < 0.0001$ ) suggesting that potentially a combination of the 2 assessment methods could be used to diagnose both established (FibroScan®) and active (ELF™) liver fibrosis (see Figure 26).

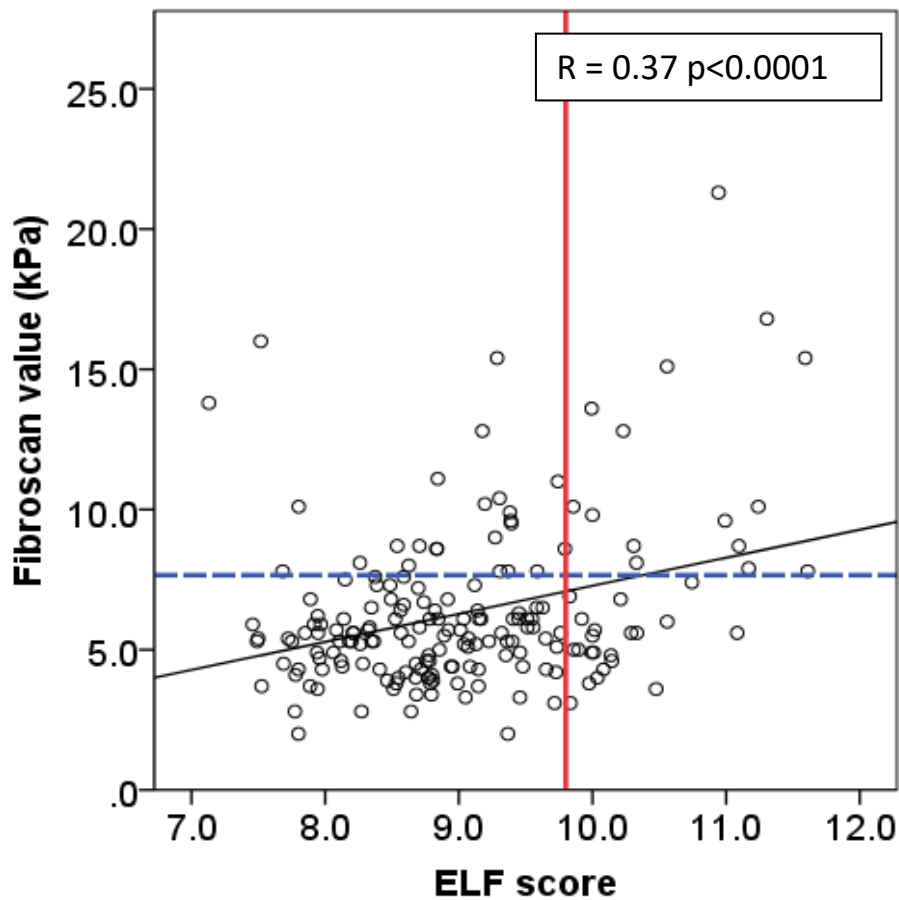


Figure 26: Association between FibroScan® and ELF score™ in the whole SCD cohort.

The horizontal reference line indicates an abnormal FibroScan® result ( $\geq 7.66$  kPa Friedrich–Rust *et al* (Friedrich-Rust, *et al* 2008)). The vertical line indicates an abnormal ELF score™ ( $\geq 9.8$  Lichtin角度 *et al* (Lichtinghagen, *et al* 2013))

### 7.3.4 Enhanced Liver Fibrosis Score

The enhanced liver fibrosis score (ELF score™) has been devised to screen for active, or to monitor ongoing fibrosis (Rosenberg, *et al* 2004). ELF score™ was calculated using serum from steady state samples, contemporaneous with transient elastography. ELF score™ results were available in 202 patients. 84 (42%) were male, 58% female. Age ranged from 17 to 72 (mean 34.6). 140 (69%) patients had SCA, 51 (25%) Hb SC and the remainder Hb Sβ<sup>+</sup>. The Hb Sβ<sup>+</sup> patients were not included in sub-group analysis due to low patient numbers. There was a significant association between ELF score™ and increasing age when corrected for sickle genotype ( $\beta = 0.2$ ,  $p = 0.005$ ) shown in Figure 27.

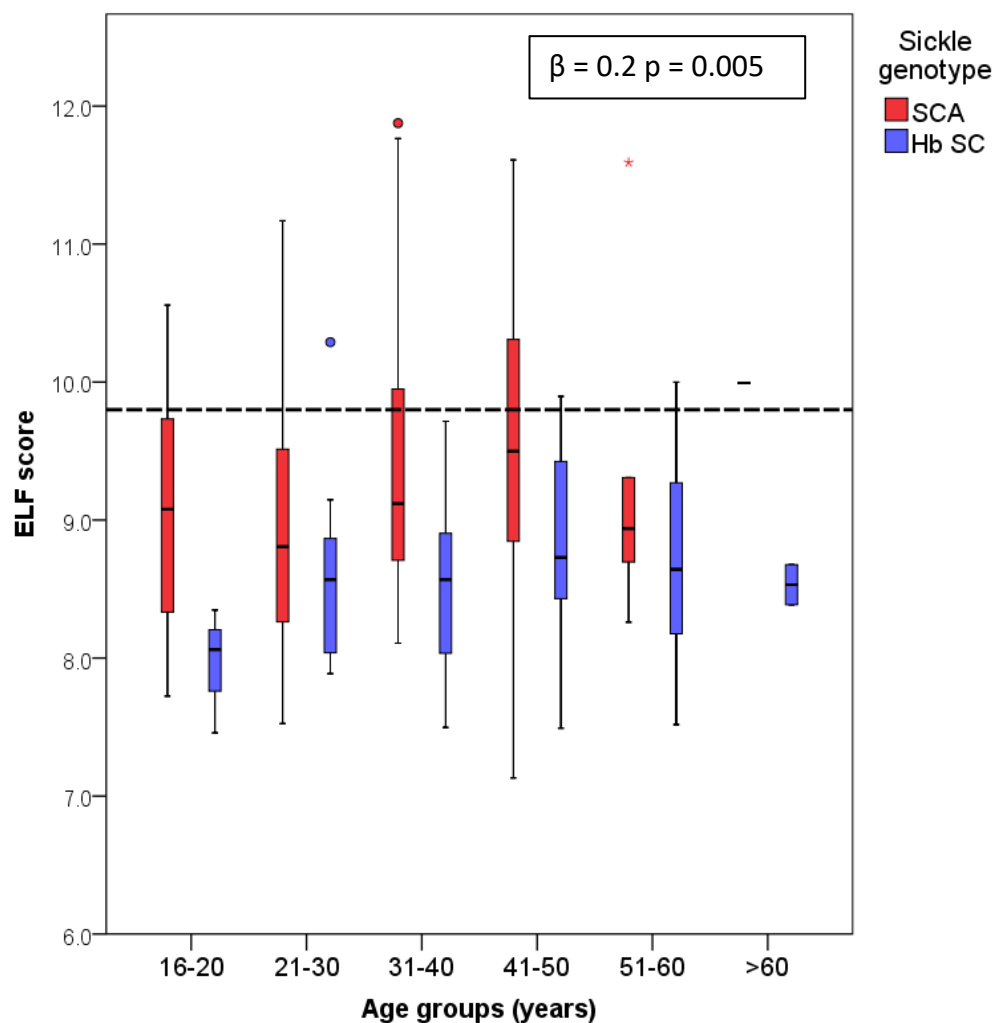


Figure 27: Effect of age and sickle genotype on ELF score™ - current presence of fibrosis

Area above horizontal limit line indicates abnormal ELF score™ ( $\geq 9.8$  Lichtin角度 *et al* (Lichtin角度, *et al* 2013))



There was no significant effect of gender on ELF score™, with or without correction for sickle genotype. Patients with SCA had significantly higher mean ELF scores™ than the Hb SC cohort (9.2 vs. 8.6 p <0.0001) (see Table 36 and Figure 27).

Table 36: The range and mean ELF score™ results for whole cohort and SCA and Hb SC subgroups.

Number and percentage of patients in each group with each separate stage of fibrosis according to iQur™, Lichtin角度 *et al* (Lichtin角度, *et al* 2013) and Parkes *et al* (Parkes, *et al* 2010). p value indicates significant difference between the mean ELF score™ for SCA and Hb SC sub-groups

ELF score	Whole cohort n = 202 (%)	SCA n = 139 (%)	Hb SC n = 51 (%)	p value
<b>Mean (range)</b>	9.1 (7.1 – 11.9)	9.2 (7.1 – 11.9)	8.6 (7.5 – 10.3)	<b>&lt;0.0001</b>
<b>iQur™</b>				
None/mild ( $\leq 7.7$ )	8 (4)	3 (2)	5 (10)	
Moderate (7.8 – 9.7)	154 (76)	100 (72)	43 (84)	
Severe ( $\geq 9.8$ )	40 (20)	36 (26)	3 (6)	
<b>Lichtin角度 <i>et al</i> (Lichtin角度, <i>et al</i> 2013)</b>				
None/mild ( $\leq 9.7$ )	169 (84)	109 (78)	49 (96)	
Moderate (9.8 – 11.2)	28 (14)	25 (18)	2 (4)	
Severe ( $\geq 11.3$ )	5 (2)	5 (4)	0 (0)	
<b>Parkes <i>et al</i> (Parkes, <i>et al</i> 2010) (risk of developing fibrosis)</b>				
None/low ( $\leq 8.34$ HR = 2.1)	44 (22)	27 (19)	16 (31)	
Moderate (8.35 – 10.42 HR = 5.1)	143 (71)	97 (70)	35 (69)	
High ( $\geq 10.43$ HR = 75)	15 (7)	15 (11)	0 (0)	

HR = Hazard ratio

The numbers of patients in the whole cohort and sub-groups with moderate or severe fibrosis varies dramatically depending on which range is chosen. These ranges were derived using the hepatitis C positive population, the manufacturers range and Lichtin角度 *et al* looking at a cross section of this patient group and comparing ELF™ results to liver biopsies whereas Parkes *et al* used longitudinal data and their end-point was the development of liver fibrosis. The relevance of either of these ranges in SCD is unclear and emphasises the importance of validating this test in the SCD cohort given these highly varied results.

The manufacturer's value ranges appear to indicate that 96% of patients in our whole cohort have moderate or severe fibrosis (98% of SCA patients). Lichtin角度 *et al* feel that the

manufacturer's value range overestimates the prevalence of fibrosis in their 2013 review of a large patient cohort, and propose a different range which would make the prevalence of moderate or severe fibrosis 16% in our cohort (22% in SCA patients). Parkes *et al* score is different: it looked instead at the predictive value of ELF score™ and looked at longitudinal results. Using the Parkes scoring system 78% of patients in our cohort (81% of patients with SCA) have a moderate or high risk of developing fibrosis in the future. Further longitudinal data will be required to see if ELF™ is predictive of outcome in sickle liver disease and whether the same results of the Parkes scoring system are seen in SCD as in Hepatitis C.

In SCA patients ELF score™ correlated strongly (using linear regression) with serum liver function tests, although less strongly than FibroScan® result (Albumin  $R = -0.30$ ,  $p < 0.0001$ , AST  $R = 0.39$ ,  $p < 0.0001$ , ALP  $R = 0.25$ ,  $p = 0.003$ , GGT  $R = 0.28$ ,  $p = 0.001$ , conjugated bilirubin  $R = 0.36$ ,  $p < 0.0001$ ). A significant positive correlation was seen with ELF score™ and LDH ( $R = 0.26$ ,  $p = 0.002$ ) and a negative correlation with Hb ( $R = -0.25$ ,  $p = 0.004$ ). In contrast to FibroScan® a significant negative correlation was also seen with HbF levels ( $R = -0.24$ ,  $p = 0.01$ ). These results are summarised in Figure 28.

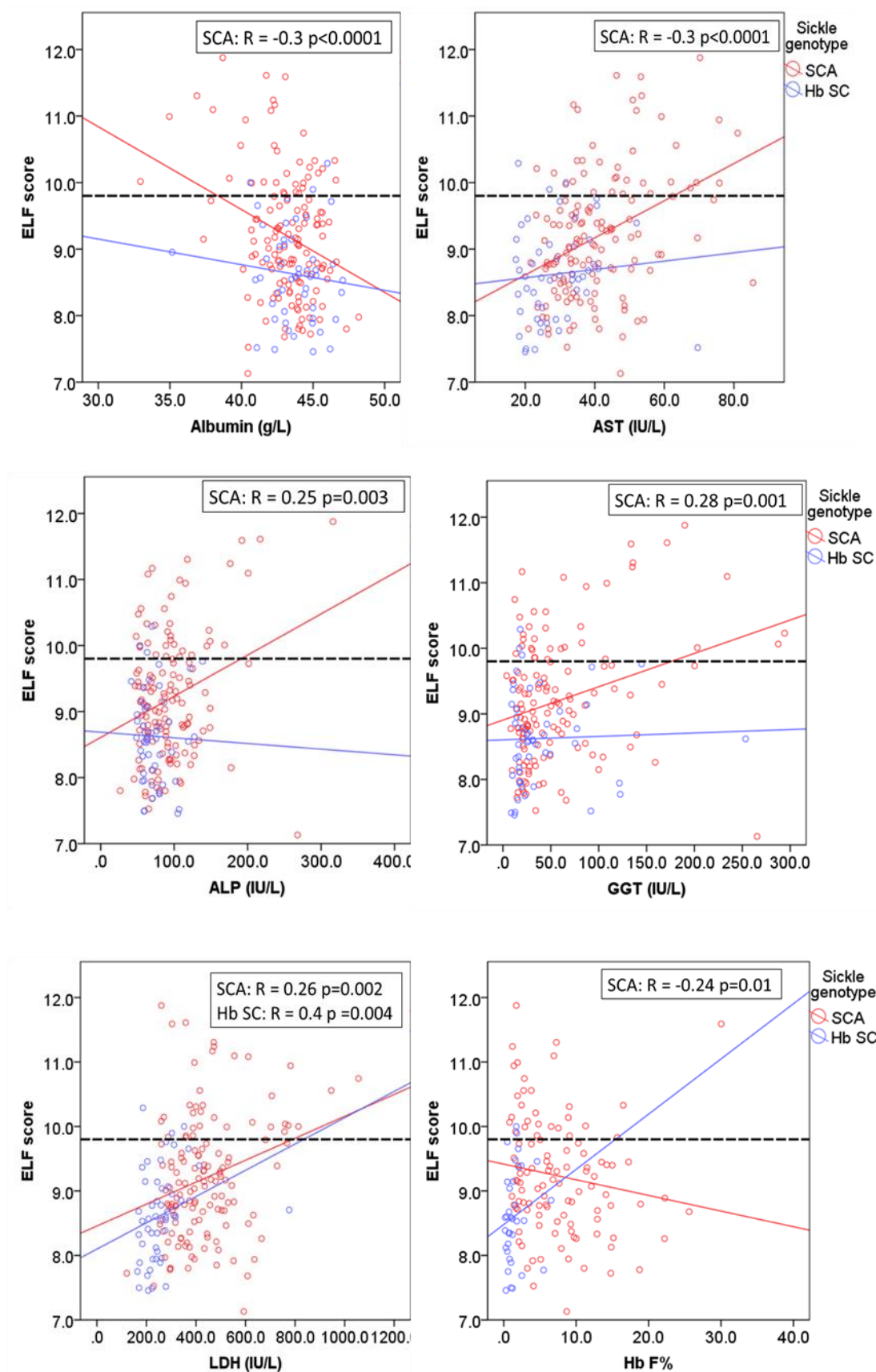


Figure 28: Graphs show the relationship between ELF score™ value, liver function tests, LDH and HbF. Significant correlations are seen in SCA with Albumin, AST, ALP, GGT, LDH and HbF. In Hb SC the correlations were only significant for LDH and Hb. The horizontal reference line indicates an abnormal ELF score™ result ( $\geq 9.8$  Lichtin角度 *et al* (Lichtin角度, *et al* 2013))

In the Hb SC group there were no significant correlations with ELF score™ and serum liver function tests. This may be a reflection of the fact that very few patients with Hb SC have serum liver function tests outside the normal range (see Table 34). However significant associations were seen between ELF score™ and LDH ( $R = 0.40$ ,  $p = 0.004$ ) and Hb level ( $R = -0.31$ ,  $p = 0.01$ ). This again supports the hypothesis that haemolysis is involved in the pathogenesis of sickle hepatopathy, however in contrast to FibroScan® sub-dividing the population dependant on LDH less than or greater than 550 IU/L did not result in a significant difference between the 2 groups.

### **7.3.5 Standard Imaging and Liver size**

After discussion with the liver radiologists at King's College Hospital, it was decided that MRI would be chosen radiological method for assessing liver size in the SCD population. This was due to it being a) the most accurate method of assessing liver size and b) reducing radiation exposure for the patients. Liver volumes were available in 30 of our patient cohort and were analysed from pre-existing MRI scans performed on our cohort. 20 patients had SCA, 9 Hb SC and 1 Hb Sβ<sup>+</sup>. 16 patients were male and 14 patients were female. Uncorrected liver size ranged from 1152 to 3482 ml (mean 2144 ml), with corrected liver size (by patient's weight) ranging from 18.2 to 65.8 ml/kg (mean 32.7 ml/kg). This is larger than the corrected mean liver size of healthy subjects (22.8 ml/kg) in the review by Andersen *et al* (Andersen, *et al* 2000). Patients with SCA had a mean liver volume of 36.14 ml/kg (range 22.5 to 65.8 ml/kg), significantly ( $p = 0.002$ ) larger than patients with Hb SC (mean 24.4, range 18.2 to 33.5 ml/kg). Age was not associated with corrected hepatic volume (even when corrected for genotype). Male patients had significantly larger mean corrected hepatic size (42.0 ml/kg) compared to females (32.2 ml/kg)  $p = 0.02$ . Due to the small sample size, further analyses were not feasible using this parameter.

### **7.3.6 Role of transfusion and iron overload**

The indications for transfusion in SCD have expanded (Drasar, *et al* 2011) leading to an increase in the incidence of hepatic siderosis seen in this patient group, despite improvements in screening (Drasar, *et al* 2012) and increased availability of chelation, including oral

preparations. In our cohort 49% of patients had received at least 1 unit of blood since the beginning of our records (1<sup>st</sup> January 1991). Mean units transfused were 22.6 (range 0 -997 units). This includes a mixture of both exchange transfusions (automated and manual) and simple top-up transfusions, with an increasing number of patients having received blood via both methods. 72 patients had received greater than 20 units top-up transfusion, which has been shown to be associated with liver iron concentrations  $\geq 7$ mg/g dry weight (Drasar, *et al* 2012) (the threshold for commencing chelation treatment). Serum ferritin levels are commonly used for screening iron overload and the monitoring of chelation therapy. In our population it ranged from 6.9 to 9873.1 ng/ml (mean 415.6 ng/ml). 45 of our cohort (8%) had serum ferritin values of greater than 1000 ng/ml, ferritin values above this level are used as a screening test indicating potentially significant iron overload. Liver iron concentration (LIC, as measured by FerriScan<sup>TM</sup>) was available in 8% (43 patients) of our cohort, with a range of 0.6 to 43 mg/g dry weight (mean 10.5).

All markers of iron loading appeared to be correlated significantly with FibroScan® values, when corrected for sickle genotype (serum ferritin  $\beta = 0.25$ ,  $p < 0.0001$ , total top-up units  $\beta = 0.22$ ,  $p = 0.001$ , total units transfused  $\beta = 0.25$ ,  $p < 0.0001$  and LIC  $\beta = 0.32$ ,  $p = 0.046$ ). See Figure 29. The borderline  $p$  value for the LIC data is influenced by the comparatively small number of patients who have received this investigation.

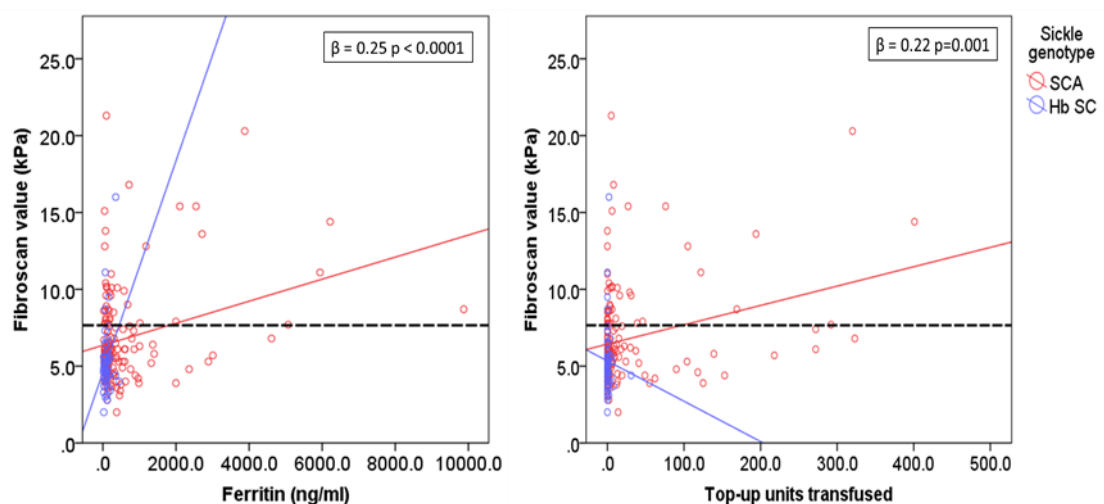


Figure 29: Associations between markers of iron loading and FibroScan® values. Liver iron concentration not shown as only 1 Hb SC patient had FerriScan<sup>TM</sup> performed

ELF score™ was correlated significantly with serum ferritin ( $\beta = 0.25$ ,  $p < 0.0001$ ), total top-up units ( $\beta = 0.24$ ,  $p = 0.001$ ) and total units transfused ( $\beta = 0.24$ ,  $p = 0.001$ ). See Figure 30. LIC was not significantly correlated with ELF score™.

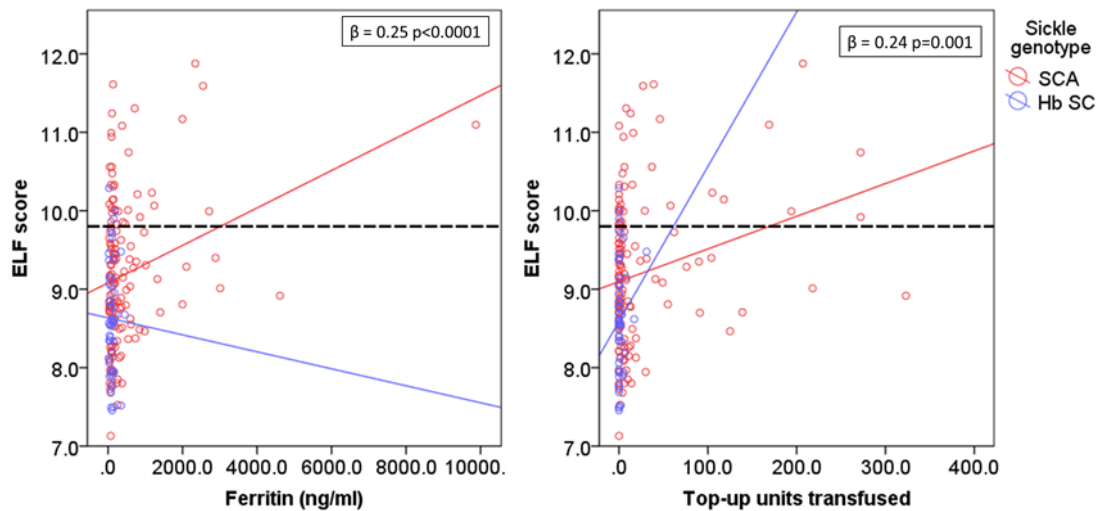


Figure 30: Associations between markers of iron loading and ELF score™ values.

Liver iron concentration not shown as only 1 Hb SC patient had FerriScan™ performed

### 7.3.7 HMOX1

As discussed in 7.1.8, heme oxygenase levels have been implicated in the development of sickle hepatopathy in a mouse model. The [GT] repeats in the promoter were genotyped by microsatellite approach. Results were generated in 299 patients of the total cohort. 196 patients (54%) had SCA, 87 (45%) Hb SC and 15 (12%) Hb S $\beta^+$ . Mean repeat number was 32.5 (range 24 to 41). Sickle genotype was not significantly associated with repeat number, showing that our dataset is not compromised by population stratification.

There were no significant associations between *HMOX1* repeat number and any serum liver function test (after correction for sickle genotype). There were also no significant associations with markers of haemolysis. No effect was seen from *HMOX1* repeat number and FibroScan™ result or ELF score™. Unlike the mouse model of sickle liver disease, our results suggest that the *HMOX1* promoter polymorphism does not play a significant role in liver dysfunction in SCD. However, we are limited by a relatively small population and a clinically heterogenous group of patients.

### **7.3.8 Screening Tool for Liver Disease in Sickle Cell Disease**

Unfortunately, chronic liver disease in SCD has heterogeneous presentations and often presents when in an advanced stage where intervention is limited, therefore a screening tool that could be routinely applied in clinic would be worth developing. Although initial results of non-invasive methods of assessing fibrosis (FibroScan® and ELF™) appear promising they are not routinely available in all centres. Therefore clinical and biological factors should be taken into account to target patients for further investigation. Due to the morbidity and ethical considerations of doing liver biopsies in SCD when not clinically indicated it was decided to use abnormal FibroScan® levels and ELF scores™ as a surrogate for liver biopsy proven fibrosis (the gold standard used in hepatitis C studies). The utility of screening tests for sickle hepatopathy was examined based on the presence of liver fibrosis as determined by an abnormal FibroScan® result or ELF™ score as described below. The numbers of true positives (a), false positives (b), false negatives (c) and true negatives (d) were calculated. Sensitivity ( $a/[a+c]$ ), specificity ( $d/[b+d]$ ), positive ( $a/[a+b]$ ) and negative ( $d/[c+d]$ ) predictive values were assessed using the binary classification test with the outcome measure of presence or absence of fibrosis.

#### **7.3.8.1 Abnormal FibroScan® as Marker for Liver Fibrosis and Abnormal Liver Function Tests as Screening Tool**

FibroScan® has published data for use as a surrogate of histologically diagnosed fibrosis (Koh, *et al* 2013, Voskaridou, *et al* 2010) in SCD, although the number of patients with histological data in this study was small. The ranges for abnormal values were taken from the meta-analysis (Friedrich-Rust, *et al* 2008) published by Friedrich-Rust *et al*, with the target to select patients with either moderate or severe fibrosis (FibroScan® result of 7.66 kPa and above). Sickle genotype and the number of abnormal liver function tests (AST, ALP, conjugated bilirubin, GGT and albumin), number of liver function tests greater than 1.5x the upper limit of normal, and the number of liver function tests twice the upper limit of normal were examined for prediction of FibroScan® result.

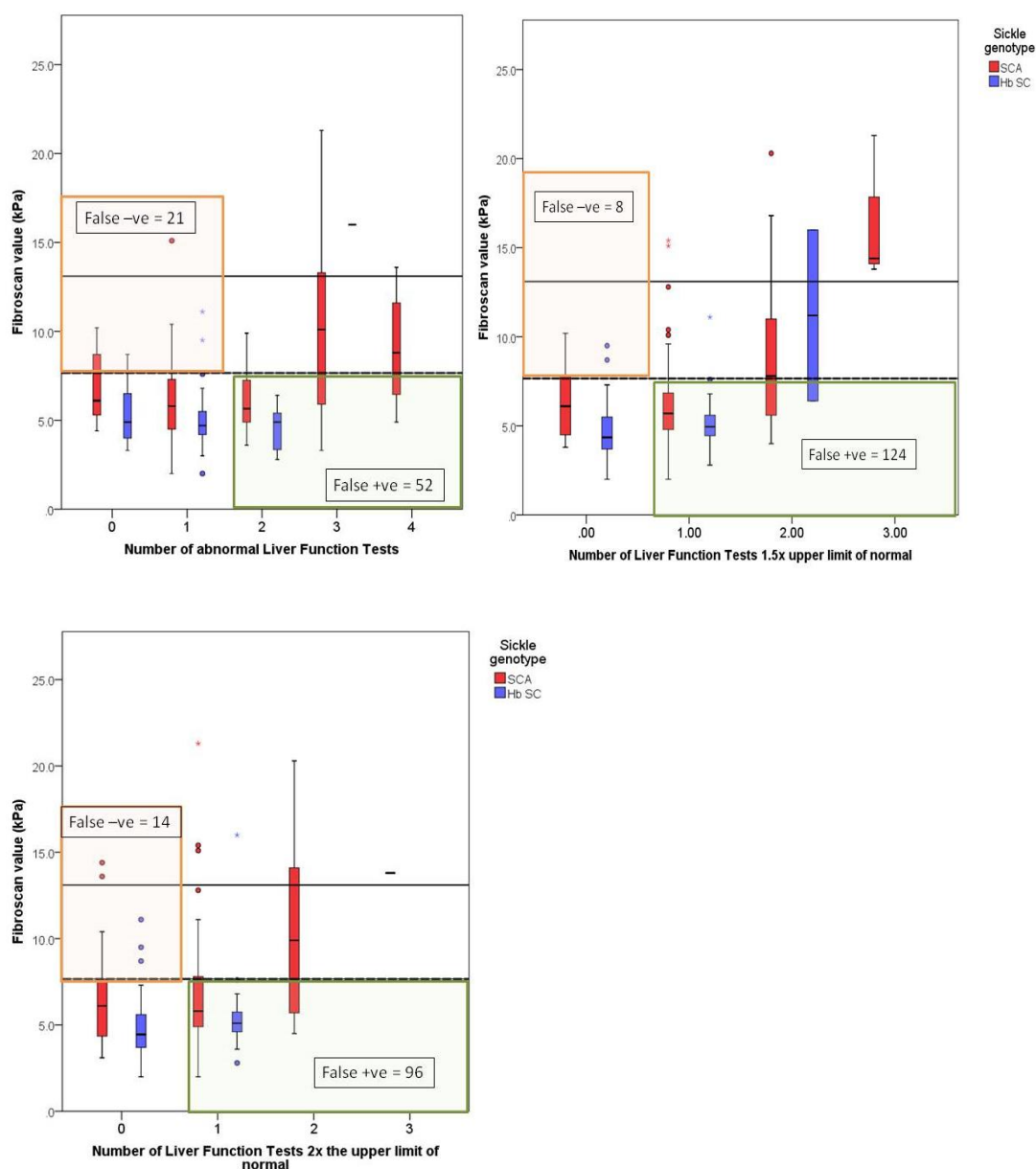


Figure 31: Abnormal FibroScan ® result stratified by sickle genotype and a) number of abnormal liver function tests, b) number of liver function tests 1.5x upper limit of normal and c) number of liver function tests 2x the upper limit of normal.

The lower horizontal reference line indicates moderate fibrosis, the upper severe fibrosis ( $\geq 7.66$  and  $\geq 13.1$  kPa respectively Friedrich–Rust *et al* (Friedrich-Rust, *et al* 2008))

Using **2 or more abnormal LFTs** (or abnormal albumin) as a screening test had a **sensitivity of 54%** (95%CI 39 – 69%) with a **specificity of 69%** (95%CI 62 – 76%). **Positive predictive value was 32%** but **negative predictive value was 85%**. Using **1 or more LFT 1.5x upper limit of normal** (or abnormal albumin) as a screening test; **sensitivity was 82%** (95%CI 69 – 92%) and **specificity 57%** (52 – 63%). **Positive predictive value was 23%** but **negative predictive value was 95%**. Using **1 or more LFTs 2x upper limit of normal** (or abnormal



albumin) as a screening test; **sensitivity was 70%** (95%CI 54 – 82%) and **specificity was 43%** (35 – 51%). **Positive predictive value was only 25%** and **negative predictive value was 84%**. These results are summarised in Figure 31.

Therefore using 1 or more LFT 1.5x upper limit of normal (or abnormal albumin) as a screening test appears to give the most favourable negative predictive value, although clearly clinical factors also need to be considered. The “false negative” patients missed by screening were either over 40 years old or had LDH values greater than or equal to 550 IU/L (more than 1 standard deviation above the mean for our population).

#### **7.3.8.2 Abnormal ELF™ Score as marker for liver fibrosis and Abnormal Liver Function Tests as Screening Tool**

There is no published data on the use of ELF™ score in SCD. However, the potential for prediction of active or the future development of fibrosis makes it an attractive outcome measure as it could potentially enable earlier detection of fibrosis and therefore earlier intervention.

Using the Lichtin *et al* (Lichtin *et al* 2013) ranges (moderate and severe fibrosis ELF™ score of 9.8 and above) and using **2 or more abnormal LFTs** (or abnormal albumin) as a screening test had a diagnostic **sensitivity of 60%** (95%CI 43 - 74%) and a **specificity of 70%** (95%CI 62 – 77%). The **positive predictive value was 34%** but the **negative predictive value was 87%**. Using **1 or more LFT 1.5x upper limit of normal** (or abnormal albumin) as a screening test; **sensitivity was 88%** (95%CI 74 – 96%) and **specificity 28%** (21 – 35%). **Positive predictive value was 24%** but **negative predictive value was 90%**. Using **1 or more LFTs 2x upper limit of normal** (or abnormal albumin) as a screening test; **sensitivity was 79%** (95%CI 63 – 90%) and **specificity was 46%** (38 – 54%). **Positive predictive value was only 28%** and **negative predictive value was 89%**. These results are summarised graphically in Figure 32 and are comparable to those found with FibroScan ®.

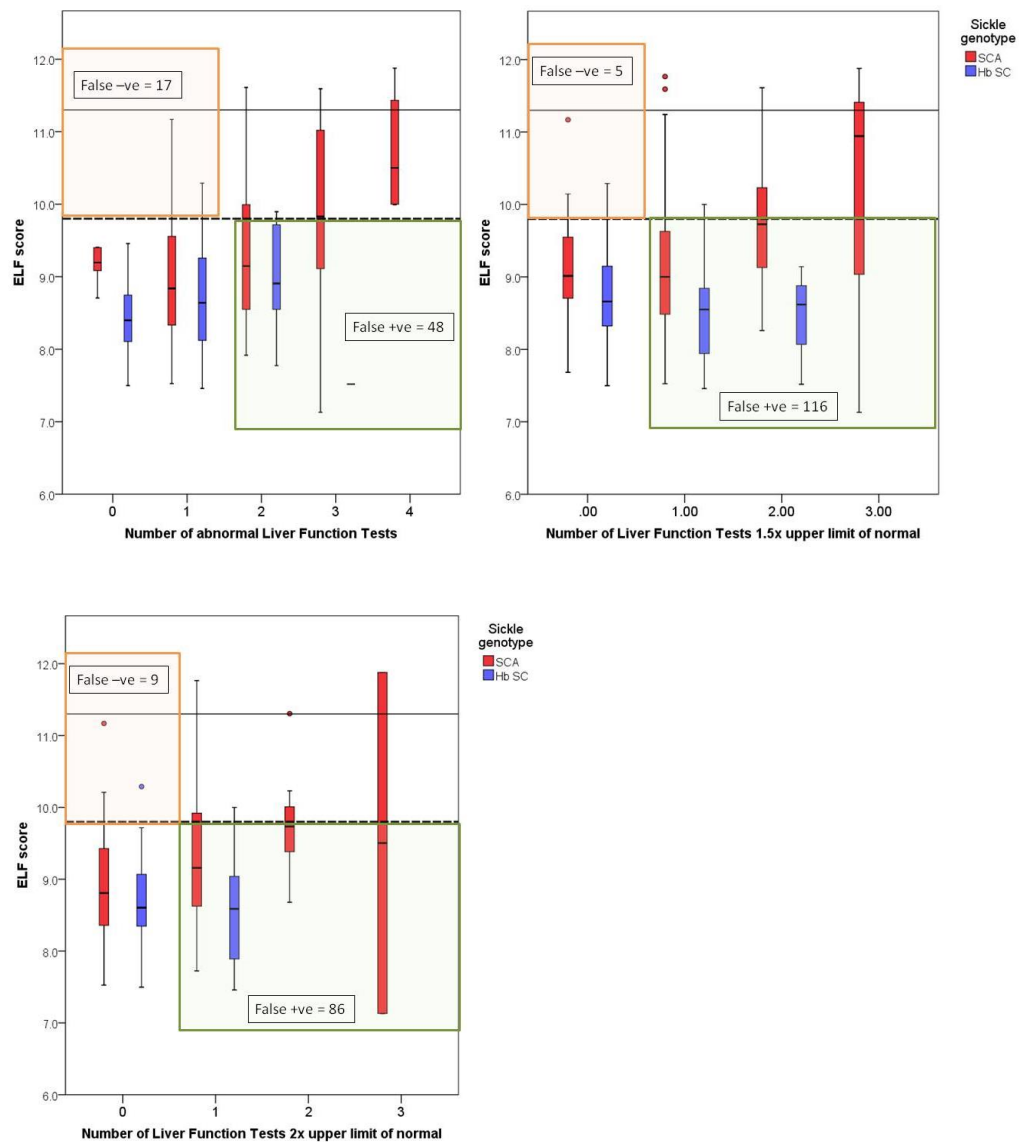


Figure 32: Abnormal ELF score <sup>TM</sup> stratified by sickle genotype and a) number of abnormal liver function tests, b) number of liver function tests 1.5x upper limit of normal and c) number of liver function tests 2x the upper limit of normal.

The lower horizontal reference line indicates moderate fibrosis, the upper severe fibrosis ( $\geq 9.8$  and  $\geq 11.3$  respectively Lichtin *et al* (Lichtin *et al* 2013))

ELF score <sup>TM</sup> has the advantage as a screening test that it requires no specialist training to perform, unlike FibroScan <sup>®</sup>. However there are ongoing cost implications, particularly in view of the high false positive rate whichever pre-screening test is applied.

As with FibroScan <sup>®</sup> using 1 or more LFT 1.5x upper limit of normal (or abnormal albumin) as a screening test appears to give the most favourable negative predictive value. Unlike FibroScan <sup>®</sup>, ELF <sup>TM</sup> showed no clear pattern with the “false negative” patients with regard to age or severity of haemolysis.

The Parkes *et al* ranges are based on longitudinal follow-up of hepatitis C patients. An ELF™ score of 8.34 and above in this study (of hepatitis C patients) was predictive of at least a moderate risk of liver related clinical morbidity or mortality. The strongest predictive effect was seen in the severe group i.e. ELF™ score of 10.42 and above and this limit was therefore selected for assessment of LFTs as a screening tool.

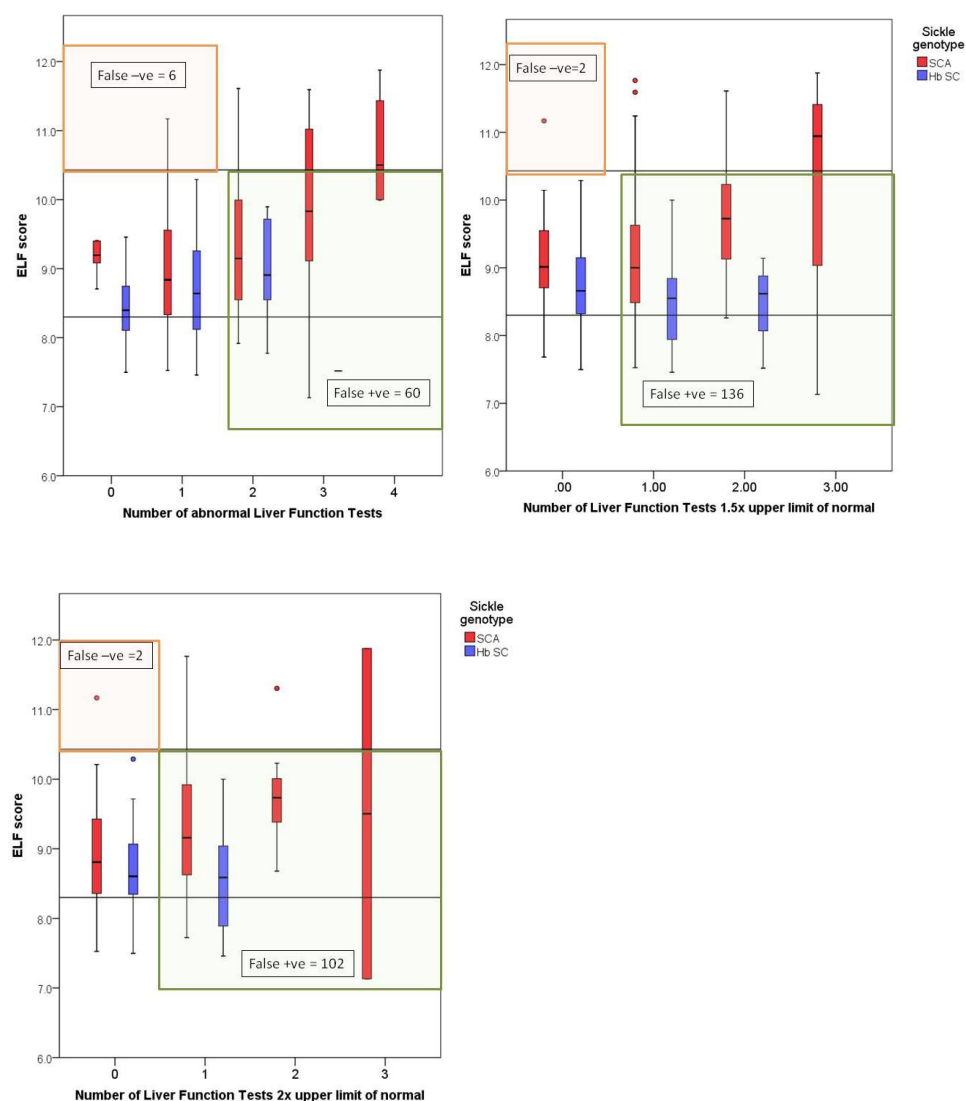


Figure 33: Abnormal ELF score™ stratified by sickle genotype and a) number of abnormal liver function tests, b) number of liver function tests 1.5x upper limit of normal and c) number of liver function tests 2x the upper limit of normal.

The lower horizontal reference line indicates moderate fibrosis, the upper severe fibrosis ( $\geq 8.3$  and  $\geq 10.43$  Parkes *et al* (Parkes, *et al* 2010))

Using **2 or more abnormal LFTs** (or abnormal albumin) as a screening test had a diagnostic **sensitivity of 68%** (95%CI 44 - 87%) and a **specificity of 67%** (95%CI 60 – 74%). The **positive predictive value** was low (**18%**) but the **negative predictive value** was **95%**. Using **1 or more LFT 1.5x upper limit of normal** (or abnormal albumin) as a screening test; **sensitivity** was **90%** (95%CI 67 – 98%) and **specificity 26%** (20 – 33%). **Positive predictive value** was **11%** but **negative predictive value** was **96%**. Using **1 or more LFTs 2x upper limit of normal** (or abnormal albumin) as a screening test; **sensitivity was 90%** (95%CI 67 – 98%) and **specificity was 44%** (37 – 52%). **Positive predictive value** was only **14%** and **negative predictive value** was the highest at **98%**. These results are summarised in Figure 33.

Using these limits for ELF score <sup>™</sup> makes the assumption that sickle hepatopathy will behave in the same way as Hepatitis C fibrosis, and therefore this should be validated prospectively prior to being put into practice as a diagnostic or screening tool.

#### **7.3.8.3 Further Investigation of Screened Population**

Although using abnormal liver function tests as a basic screening tool for sickle hepatopathy appears to have a strong negative predictive value for all limits of LFTs and modalities chosen, there is an argument to be made that all SCD patients should undergo specialist screening for sickle hepatopathy on a regular basis. In our cohort, we have a prevalence of at least moderate fibrosis in 23% of patients (29% in SCA) using FibroScan ® or 16% of patients (22% in SCA) using ELF score <sup>™</sup>. All patients with Hepatitis C (a comparable cause of fibrotic liver disease) undergo annual screening FibroScans in our centre; should the same facility not be available for patients with sickle cell disease? This technology is not available at all hospitals. ELF score <sup>™</sup> could potentially provide an alternative screening process for patients away from tertiary liver centres, with patients having abnormal values then being referred in for further investigation. This has cost implications and, as little longitudinal data is available in SCD, may be difficult to justify. It is here that using abnormal LFT limits can have a function, with a lower threshold for referral in patients over the age of 40, particularly those with a “haemolytic” phenotype. Iron overload also appears to be significant in having abnormal FibroScan ® and ELF score results and therefore should be addressed prior to other interventions.

Figure 34, below, shows a suggested patient pathway for further investigation of patients screened as “high risk” using liver function tests. This pathway aims to capture patients when they still have moderate fibrosis when intervention can make the most clinical impact and ensures prompt referral to appropriate specialist services. As these patients are seen in conjunction with a hepatologist with a specialist interest in SCD appropriate interventions can then be suggested back to the local hospital, including further monitoring if required. Further prospective investigation needs to be performed to evaluate this tool prior to routine clinical use.

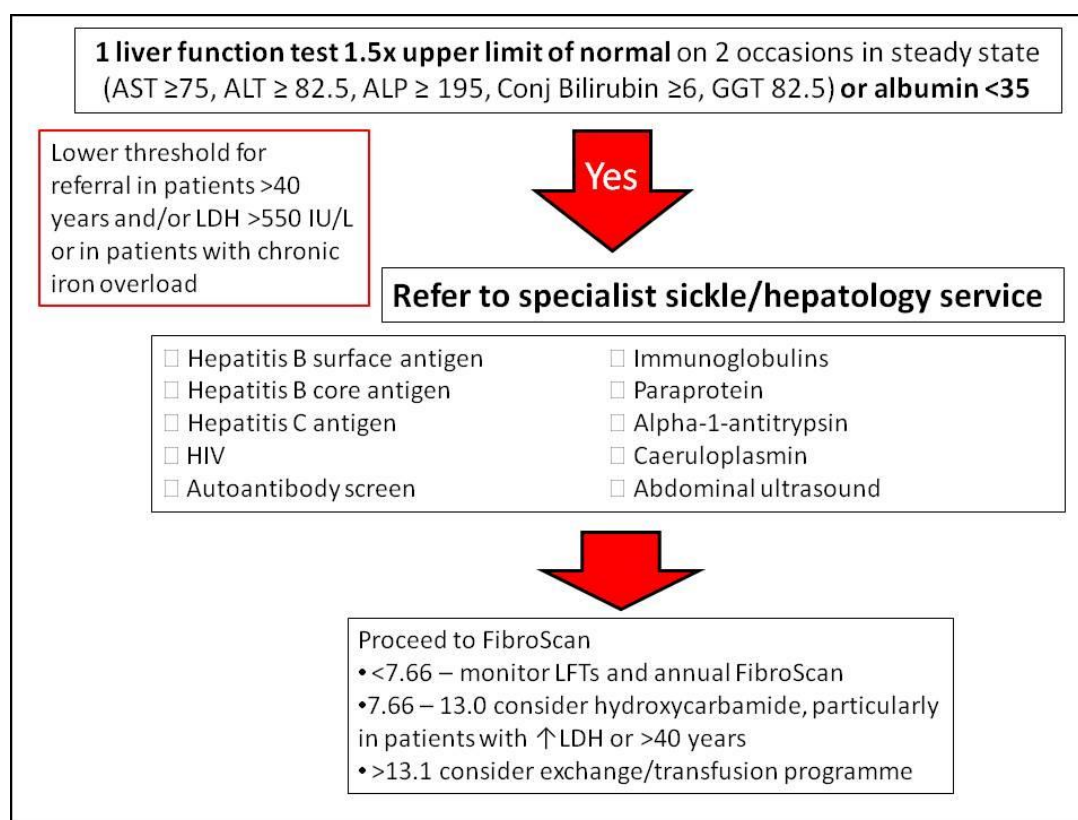


Figure 34: Patient pathway for investigation of sickle hepatopathy including a suggested order for general and specialist investigations.

## 7.4 Conclusion

We have described the prevalence of liver dysfunction in our sickle cell disease population using both standard tests (serum liver function) and novel non-invasive methods (transient elastography and ELF™ score) that are coming into mainstream practice in other causes of liver fibrosis. Liver dysfunction appears to be a potentially significant problem amongst the SCD population, with 38% having at least 1 abnormal serum liver function test, increasing to 48% in the SCA subgroup. FibroScan® shows a significant prevalence of underlying liver fibrosis in our population, with at least 23% of our patients having moderate or severe fibrosis using this method of assessing fibrosis. The ELF score™ has less clearly defined ranges of normality and abnormality, with different values indicating abnormality depending whether ongoing fibrosis or future liver morbidity/mortality is the defined outcome. Depending on the range used the prevalence of ongoing fibrosis (or at least extracellular matrix turnover) ranges from 16% of patients having moderate/severe fibrosis to a somewhat shocking 96% of the whole cohort. In the light of the FibroScan® results obtained in parallel, the ELF score™ data seem to represent an overestimate of established fibrosis, and indeed a review of the literature of ELF score™ in Hepatitis C appears to indicate that the normal ranges given by the company are in the process of being revised in this setting. Unsurprisingly, all measures of liver function including serum liver tests, transient elastography and ELF score™ are significantly higher in SCA compared with Hb SC, indicating the more severe phenotype of this condition. Both FibroScan® and ELF™ score correlate significantly with both abnormal liver function tests and haemolytic markers in the SCA population. The correlation with haemolytic markers adds further evidence to the theory that there is an underlying role of haemolysis in the pathogenesis in sickle liver dysfunction.

We have also examined the role of liver imaging in assessing the prevalence of liver disease in our SCD patients. Our cohort had significantly larger corrected liver size (as assessed by MRI scan) compared with previously published healthy subjects. However, as these scans were only indicated (and therefore performed) in patients in whom liver pathology was suspected, this may not reflect the distribution of size across our cohort. Liver radiologists at King's College Hospital (Dr Pauline Kane) hypothesise that liver size is a significant negative outcome measure in SCD but this will have to be validated in a larger population of patients, both with and without

liver dysfunction. We can then correlate these data with our other outcome variables including markers of liver function and haemolysis.

Transfusion and iron overload is significantly associated with the development of liver fibrosis (as measured by FibroScan® and ELF™ score) in this setting. This has a potentially significant treatment implication in that we should be potentially be more aggressive in our screening and chelation of our patients to attempt to reduce one of the few risk factors for liver disease for which we have a useful intervention. Longitudinal measurements (FibroScan® and ELF score™) of patients throughout their transfusion programmes and chelation treatment could indicate whether the removal of iron from the liver actually makes a difference to outcome in the form of liver fibrosis and cirrhosis.

We also examined the role of a candidate gene in the development of liver fibrosis in SCD. *HMOX1* has been associated with sickle hepatopathy, in the transfused sickle mouse, but was not found to be associated in our human cohort. This may be related to our study being underpowered to show this association.

Given the current and future disease burden from sickle liver dysfunction indicated by the above we have attempted to develop a screening score for liver dysfunction using presence of moderate and severe fibrosis (FibroScan ®) and active ongoing extra cellular matrix turnover (ELF™ score) as our end-points (in view of liver biopsy being contraindicated in our population of patients). This can be utilised in hospitals which do not have open access to FibroScan ® as a screening modality. I have also outlined a potential pathway for intervention and further investigation in these patients.

In summary, sickle liver dysfunction is a multifactorial problem with underlying haemolysis as a major contributor to its pathophysiology. Liver siderosis also contributes to its pathogenesis, and aggressive iron chelation is recommended in the setting of iron overload.

The role of transient elastography and ELF score™ in monitoring liver dysfunction in SCD needs to be further validated, preferably with longitudinal, and if possible histological data. The new development of transient elastography MRI may also provide further information about the involvement of the liver in sickle cell disease.

## Chapter 8 Final Discussion and Future Directions

Sickle cell disease (SCD) presents with a variety of clinical complications, the frequency of which will increase with an aging patient population. This is confirmed by the description of our own cohort (see Chapter 3). Using this carefully characterised population of patients my thesis had the overall aim of further exploring the pathophysiology of sickle cell disease and identifying potential predictors/modifiers of disease severity. I have also explored in more detail two specific end-organ complications in SCD, namely renal and liver failure.

Factors that can impact on disease severity include laboratory variables (e.g. haemoglobin or WBC levels), genetic factors (co-inheritance of specific polymorphisms alongside the sickle gene) and treatments (hydroxycarbamide and transfusion). These factors and their influence on disease severity and the development of specific complications have been explored in detail as outlined below.

The main conclusions of my thesis are:

**Chapter 4: In order to further understand the pathophysiology of SCD we examined correlations between leukocyte telomere length and markers of haemolysis and inflammation in SCD. We postulated that the haemolytic environment of SCD would lead to shortened telomeres compared to age matched controls. This was the first time telomere length had been examined in SCD.**

Leukocyte telomere length, as measured using multiplex QPCR, has a much larger range of lengths compared to controls. Significantly, and in contrast to what we expected, longer telomeres were found in sickle cell patients compared to our control population. We had expected that the haemolytic environment and the oxidative damage in SCD would lead to increased telomere loss. Also, surprisingly, patients with SCA had significantly longer telomeres than patients with Hb SC, despite their classically more severe phenotype.

We postulate that the longer telomeres in patients with SCD relates to upregulated telomerase activity secondary to the chronic systemic inflammation, and activated leucocytes (Field, *et al* 2013, Turhan, *et al* 2002)). This suggestion is supported by the: 1) positive association of relative telomere length with WBC and neutrophil count, markers of inflammation; 2) relatively longer telomere lengths in patients with SCA when compared to Hb SC patients who have less



inflammation (Nagel, *et al* 2003); 3) significantly shorter telomeres in patients on hydroxycarbamide therapy compared to the untreated group, probably mediated through the anti-inflammatory effects of HC via suppression of WBC count and down regulation of cytokines (Lanaro, *et al* 2009).

Future work would include measurement of telomerase levels (on and off HC) to confirm the mechanism of longer telomeres in the SCD population. Telomere length could also be examined in the different cell populations (i.e. WBCs and NRBCs) to ascertain the cell source. Longitudinal measurement of telomere length in patients with SCD could also be examined, particularly in those patients pre and post commencement on hydroxycarbamide to assess the intra-patient effects of this treatment.

**Chapter 5: We evaluated the effect of the common Duffy Antigen Receptor for Chemokines (DARC) polymorphism on disease severity in SCD. We examined its effect on laboratory variables in the acute and steady-state, on the development of end-organ complications and on admission parameters (including length of stay and time to readmission).**

We confirmed previous research by other groups that Duffy negative patients with SCD have lower WBCs and neutrophil counts. We found associations with the Duffy positive phenotype and the development of leg ulcers and markers of renal impairment.

We speculate that the association with leg ulcers may be secondary to the relatively higher white cell and neutrophil counts in Duffy positive patients, potentiating inflammation and predisposing to skin infarction and infection. The association with markers of renal impairment was explored further in Chapter 6, correcting for other known influences on renal function such as the role of haemolysis and other genetic polymorphisms.

Although Duffy phenotype was not clearly associated with markers of disease severity, our study period was relatively short and future work could include a larger admission period and therefore a bigger dataset. Further work is required to validate the hypothesis that the raised WBC associated with Duffy positive phenotype could potentiate increased rates of vaso-occlusion and potentially be a risk factor for the development of leg ulceration.

**Chapter 6: Renal complications in SCD and their predictors were explored in this chapter. Associations between markers of renal impairment and potential modifiers including laboratory variables (specifically markers of haemolysis) and genetic factors (candidate genes including *DARC*, *APOL1* and *HMOX1*).**

We demonstrated significant associations between both degree and prevalence of albuminuria and all markers of increased haemolysis in patients with SCA. Significant genetic associations were found with 2 different markers of renal dysfunction. Duffy positive phenotype was associated with the development of macroalbuminuria and presence of 1 or more *APOL1* risk alleles was associated with the development of renal impairment (as measured by MDRD eGFR).

It is unclear as to whether the effect on renal function seen is a true effect of Duffy phenotype (e.g. via its effect on WBC) or whether it is a surrogate marker for another Caucasian population derived polymorphism (and purely indicates a significant degree of admixture). The significant association with MDRD eGFR and *APOL1* risk alleles is similar to what has been found with other non-sickle African derived populations with all causes of end-stage renal failure.

**Chapter 7: Definitions, methods of diagnosis and prevalence of liver disease were explored in this chapter. Potential modifiers/predictors were examined including laboratory variables, genetic factors (*HMOX1*) and therapy related complications (iron overload secondary to transfusion). Novel non-invasive methods of diagnosis were evaluated and a care pathway for this complication proposed.**

We described the prevalence of liver dysfunction in our sickle cell disease population using both standard tests (serum liver function) and novel non-invasive methods (transient elastography and ELF™ score) and have found it to be a significant problem which appears to increase with age.

Transfusional iron overload and haemolysis also appear to play a key role in its pathogenesis. We have attempted to develop a screening method for liver dysfunction using presence of moderate and severe fibrosis (FibroScan ®) and active ongoing extra cellular matrix turnover (ELF™ score) as our end-points (in view of liver biopsy being contraindicated in our population

of patients). Both FibroScan ® and ELF™ score need to be validated longitudinally in SCD as this may provide valuable predictors about the pace of the development of liver impairment in this population, as well as the value of any interventions (e.g. chelation or transfusion therapy). I have also outlined a potential pathway for intervention and further investigation in these patients which also needs to be validated prospectively.

Sickle cell disease is a challenging condition to manage due to its complexity. Control of infection and improved supportive medical care, has improved survival in newborns, emerging complications which require treatment, are now seen increasingly in adults. Despite significant advances in understanding the pathophysiology of sickle cell disease, we are still, fundamentally limited to two treatment options – blood transfusion and hydroxycarbamide. Until further therapeutic options become widely available, sickle cell disease will still be a life-limiting condition.

## Chapter 9 : References

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